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Novel Approaches to Anti-Inflammatory Agents as Therapeutics for Asthma

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Abstract: It is now a well-acknowledged fact that lung airway and tissue inflammation is a major aspect of the pathobiology of pulmonary diseases such as asthma, emphysema, adult respiratory distress syndrome, cystic fibrosis and interstitial lung diseases. Asthma incidence is increasing and standard therapies show significant flaw. Although steroid therapy plays a major role in the treatment of asthma, it suffers from a number of side-effects. Currently, new approaches directed toward the treatment of inflammation associated with pulmonary diseases have included the development of agents which have impact on T-lymphocytes, mast cells, macrophages, neutrophils and eosinophils. These cells have the ability to produce some inflammatory mediators (e.g. cytokines, leukotrienes and cyclooxygenase products) or to release degradative enzymes, toxic proteins and toxic oxygen metabolites. It has also been demonstrated that leukotrienes play an important role in the pathogenesis of asthma. Leukotriene receptor antagonists and 5-lipoxygenase and 5-lipoxygenase-activating protein (FLAP) inhibitors are likely to be the most significant new anti-asthma drugs.

This review will deal with new approaches for asthma therapy as well as discuss new anti-asthma drugs.

Keyword: Asthma, leukotrienes, leukotriene receptor antagonists, 5-lipoxygenase inhibitors, FLAP inhibitors

INTRODUCTION

Asthma is a chronic inflammatory condition characterized by bronchial hyper-responsiveness and reversible airway obstruction [1]. The primary goal of asthma management is to maintain control of the disease process by reducing symptoms and asthma exacerbations and improve lung functions. Pharmacological control of asthma can be achieved in most patients with anti-inflammatory agents such as cromones and inhaled glucocorticoids. In addition, long acting β_2 agonists (salmeterol, formoterol, bambuterol and slow release terbutalin) and theophylline have also been shown to control asthma, albeit to a lesser degree.

However, the β_2 agonists, unlike the inhaled glucocorticoids, do not reverse airway inflammation and hyper-responsiveness. In contrast to the "compounds" described above that "control" the asthmatic condition, short-acting bronchodilators function as "relievers" of asthma and rapidly reverse acute manifestations such as respiratory symptoms and airflow limitation.

The initiation and propagation of airway inflammation arises from many factors including mediators generated by resident airway cells and recruited leukocytes. Among these mediators the leukotrienes (LTs) have been shown to be one of the most important. Indeed, the idea that LTs play a significant role in bronchial asthma has been supported by three main lines of evidence: (1) cells known to be involved in asthma produce LTs. Neutrophils, monocytes, endothelial cells, and T-lymphocytes all produce leukotriene LTB_4 if stimulated with LTA_4 from other cells. Similarly, the cysteinyl LTs (LTC_4, LTD_4 and LTE_4) are produced by monocytes, eosinophils, mast cells, endothelial cells and platelets; (2) Cysteinyl LTs cause airway abnormalities that mimic those seen in asthma, i.e., bronchoconstriction, inflammatory cell filtration, increased vascular permeability, increased mucus secretion, and increased airway responsiveness; and (3) The production of LTs is increased in the airways of people with asthma [2-4].

The discovery of leukotrienes began in 1940 when Kellaway and Trethewie sensitized guinea pigs to ovalbumin [5]. Subsequent challenge with the allergen led to a slow, prolonged contractile response of the animals' intestinal and airway smooth muscles. The term "slow reactive substance" (SRS) was coined to appropriately describe the active principle involved in the contractile response. Some 20 years later, human asthmatic tissue was shown to contract in response to the same allergen in a very similar manner [6]. Thus, the term slow reacting substance of anaphylaxis (SRS-A) was born. Despite intensive research efforts, it was not until 1979 that the chemical constituents of SRS-A were identified as the cysteinyl leukotrienes (LTs), namely LTC_4, LTD_4, and LTE_4 [7,8]. Their effects mimic the pathological changes seen in asthma both *in vitro* and *in animal models*. The demonstration that leukotrienes have proinflammatory properties relevant to the pathogenesis of asthma has stimulated the development of potential therapeutic compounds to block these actions.

LEUKOTRIENE FORMATION

The leukotrienes are formed from arachidonic acid via the lipoxygenase pathway (Fig. 1). The first step involves the

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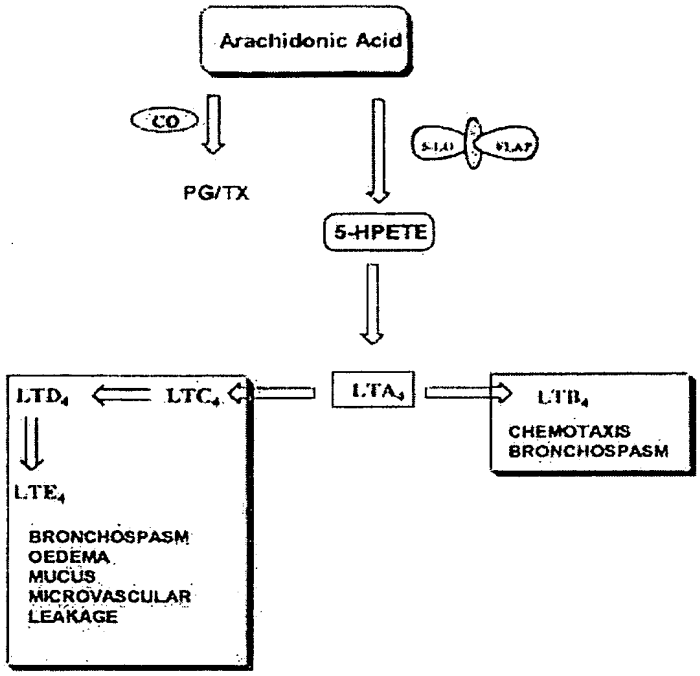


Fig. (1). The leukotriene biosynthetic pathway. Arachidonic acid, in association with 5-lipoxygenase activating protein, is converted initially to 5-hydroperoxy-eicosatetraenoic acid and then to leukotriene A_4. LTA_4 is then converted to either LTB_4 or the cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4).

action of active 5-lipoxygenase in the presence of an 18kD membrane protein called 5-lipoxygenase-activating protein (FLAP) on arachidonic acid to form an unstable intermediate, 5-hydroxy-eicosatetraenoic acid (5-HPETE), which is then converted to the epoxy leukotriene (LTA_4) [9,10]. LTA_4 may then be converted to LTB_4 or it may also be converted to LTC_4 by conjugation with glutathione. LTC_4 is then actively transported out of the cell where it is further metabolized to LTD_4 and then LTE_4. LTE_4 is either excreted unchanged in the urine or further metabolized to a number of biologically inactive intermediates, which are also excreted through urine. Investigators have used the measurement of LTE_4 in urine of the patients as a convenient estimate of leukotriene production. LTB_4 is mainly produced by neutrophils and its predominant effect is that of neutrophil chemotaxis, although it also has a lesser chemoattractant effect on eosinophils. The other leukotrienes LTC_4, LTD_4 and LTE_4 are also known as "sulphidopeptide leukotrienes or "cysteinyl leukotrienes " due to the presence of thioether-linked peptide structure [11,12]. Cysteinyl leukotrienes have been shown to be produced by eosinophils [13], mast cells [14], macrophages and monocytes [15] and basophils[16].

It is well recognized that cysteinyl leukotrienes are not synthesized and stored in these cells as is histamine in mast cells. Instead, cells that contain the leukotriene biosynthetic machinery are activated by the specific stimuli, which include the immunoglobulins IgE, IgG, endotoxin and certain phagocytic stimuli such as cytokines and chemokines. This activation causes cells to generate leukotrienes by themselves [17].

LEUKOTRIENE RECEPTORS

These mediators exert their effects through G protein-coupled receptors regulating a signal transduction pathway that ultimately causes calcium release from the cells [18]. The leukotriene receptor classification that has been developed is based on the pharmacological receptor blocking experiments, since molecular cloning of the cysteinyl luekotriene receptors has so far been unsuccessful [18-20]. However, the receptor for LTB_4 (the hydroxyleukotriene generated from LTA_4 via the action of LTA_4 hydrolase in cells such as neutrophils) has recently been cloned [21]. This may lead to success with other cysteinyl leukotriene receptors in the future. To date, two subtypes of cysteinyl leukotriene receptors have been categorized, the $cysLT_1$ and $cysLT_2$ receptors (Fig. 2).

There are two classes of receptors for leukotrienes: 1) The BLT_1 receptor (there is apparently only one type of LTB_4 receptor designated as BLT_1 and hydroxyleukotriene LTB_4 binds to this receptor) and 2) CysLT receptors 1 and 2 (cysteinyl leukotrienes bind to these receptors). In human lungs, the cysteinyl leukotrienes activate the $cysLT_1$ receptors. On the other hand the $cysLT_2$ receptor is present in human pulmonary vasculature. However, little is known about the $cysLT_2$ receptor, other than it is found in the pulmonary vascular system on endothelial cells and vascular smooth muscle. In contrast, the $cysLT_1$ receptors are principally responsible for mediating the actions of the cysteinyl leukotrienes in asthma [18-20].

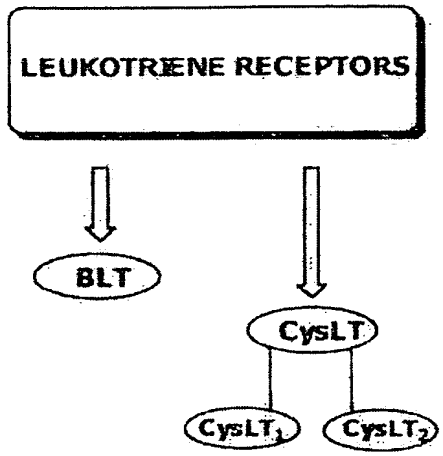


Fig. (2). Classification of leukotriene receptors.

THE RESPONSE OF ASTHMA TO LEUKOTRIENES

The cysteinyl leukotrienes have clearly demonstrated profound biochemical and physiological effects (at subnanomolar concentrations in some cases) that include induction of plasma extravasations, stimulus of mucus secretion, impairment of mucociliary transport, chemotaxis of eosinophils from the bronchial microcirculation, sensitization of the sensory nerve terminals (including neuropeptide release) and contraction and proliferation of airway smooth muscle cells [22]. In humans, inhaled LTC_4 and LTD_4 are potent bronchoconstrictors in both normal and asthmatic patients. They are approximately a thousand-fold more potent (as bronchoconstrictors) than methacholine in normal subjects and have a longer duration of action than inhaled histamine [23]. Inhaled LTE_4 has been showed to result in eosinophil chemotaxis in the asthmatic lung [24]. Furthermore, inhaled cysteinyl leukotrienes, at concentrations below the threshold for bronchoconstriction induction, induce a marked hyper-responsiveness to other inhaled agents, such as histamine and methacholine [25]. Cysteinyl leukotrienes have also been recovered after a variety of experimental challenges in asthmatic subjects who elicit clinical symptoms similar to those associated with an occurring asthmatic attack. Thus, cysteinyl leukotrienes are recovered in significant amounts in the bronchoalveolar lavage of aspirin sensitive asthmatic patients after aspirin inhalation antigen challenge and in patients subjected to cold air [26,27]. After such challenges, the levels of the LTE_4 recovered in the urine with asthmatic subjects were increased. These observations are evidences that leukotrienes are produced in the lungs of asthmatic patients and are associated with the response. These results have stimulated the development of compounds specifically designed to inhibit the production or to block the action of leukotrienes [28].

LEUKOTRIENE INHIBITION AS A TREATMENT OF ASTHMA

Leukotriene therapeutic interventions have been directed toward halting the synthesis of leukotrienes through inhibition of the action of leukotrienes with leukotriene receptor antagonists and blocked of the 5-LO or FLAP activities [4,12,29,30]. Leukotriene receptor antagonists represent the first in a new class of antiasthma drugs to be introduced into clinical practice during the last 20 years. Many placebo-controlled studies have clearly demonstrated the efficacy of cysteinyl leukotriene receptor antagonists in chronic asthma [31]. On the other hand, early studies of LTB_4 receptor antagonists have shown that they have no effect on lung function in asthmatic patients [32].

First-Generation Cysteinyl Receptor Antagonists

The first generation of cysteinyl receptor antagonists was neither selective nor potent. The list includes compounds such as FPL-55712 **(1)**, LY 171,883 **(2)** (tomelukast), CGP 35,949 **(3)**, YM-16638 **(4)**, LY-163,443 **(5)**, L-647,438 **(6)**, L-649,923 **(7)**, Ro23-3544 **(8)** and REV-5901 **(9)** (Fig. 3). FPL-55712 was one the first leukotriene receptor antagonists having been identified in the late 1970's [33]. Pretreatment of smooth muscle with FPL 55712 was shown to reduce contraction caused by the addition of antigen to guinea pig trachea and human bronchial tissue preparations. In addition, when FPL-55712 was added to the tissue preparations after antigen stimulation, it reversed antigen-induced contraction [33]. On the other hand, the first clinical asthma study was conducted in 1982 by giving nebulised FPL-55712 to 4 patients with chronic asthma. FPL-55712 had an unpleasant taste and caused a transient discomfort in the throat when given in this manner [34]. The next compound, tomelukast was identified in 1988 and found to be a competitive antagonist of LTD_4 on GP ilea and parenchyma. However, it was a noncompetitive LTD_4 antagonist on GP trachea and did not antagonize the effects of LTC_4 on GP ilea [35]. In contrast, it was effective against both LTC_4 and LTD_4 induced bronchospasm in guinea pig. The bronchospasm produced by ovalbumin in sensitized GP_5 was also blocked by tomelukast at a dose of 30mg /kg p.o. This compound also possesses bronchodilator activity [36]. In the first long term study, tomelukast at 400 mg twice a day reduced symptom scores, decreased the use of a β_2 agonist and increased FEV 1 (forced expiratory volume in one minute, an objective measure of lung function) in asthmatic patients. When a dose of 600 mg twice daily for 6 weeks was given, several patients experienced diarrhea and headache [37].

Tomelukast has also been shown to produce small but significant protective effects during the early phase allergen challenge [38, 39]. At a dose of 30 mg/kg p.o in allergic sheep, tomelukast blocked the late phase bronchospasm and significantly induced the acute reponse, but did not affect the antigen-induced fall in tracheal mucous velocity [40]. In asthmatic patients, the late phase response to allergen provocation was blocked after treatment with 75-600 mg of tomelukast BID for 5 days [41]. The structure-activity relationships in the series leading to tomelukast showed that the 2-propyl-3-hydroxy-4-acetylphenoxy unit was important for activity and 4-7 metylene unit spacer was optimal between the phenoxy and tetrazole units [42,43]. Several other analogs of this structural type including CGP 35,949 [44] ,YM-16638 and LY-163,443 [45] were reported to be orally active in animals. The orally–active compound YM-

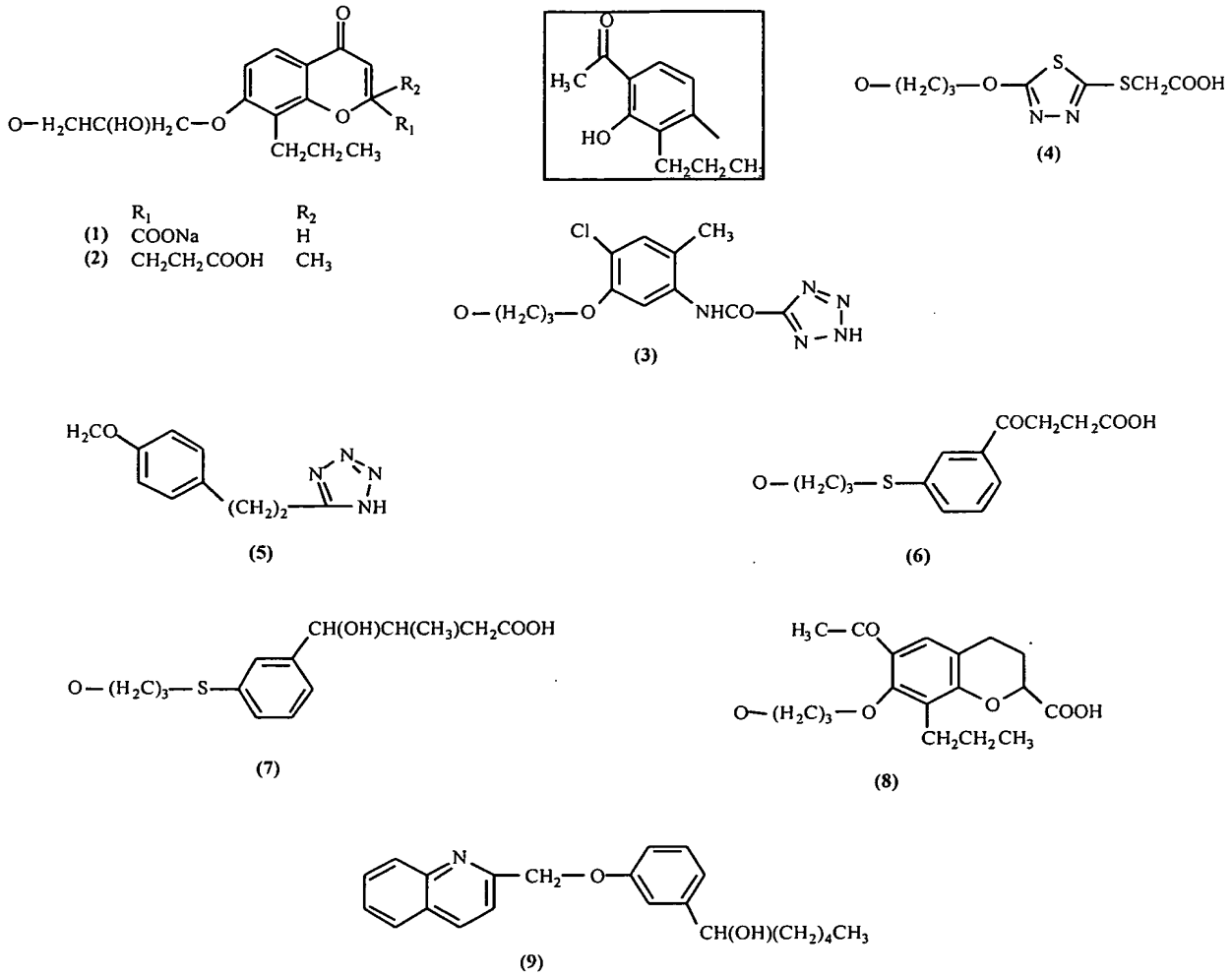


Fig. (3). First-generation cysteinyl receptor antagonists

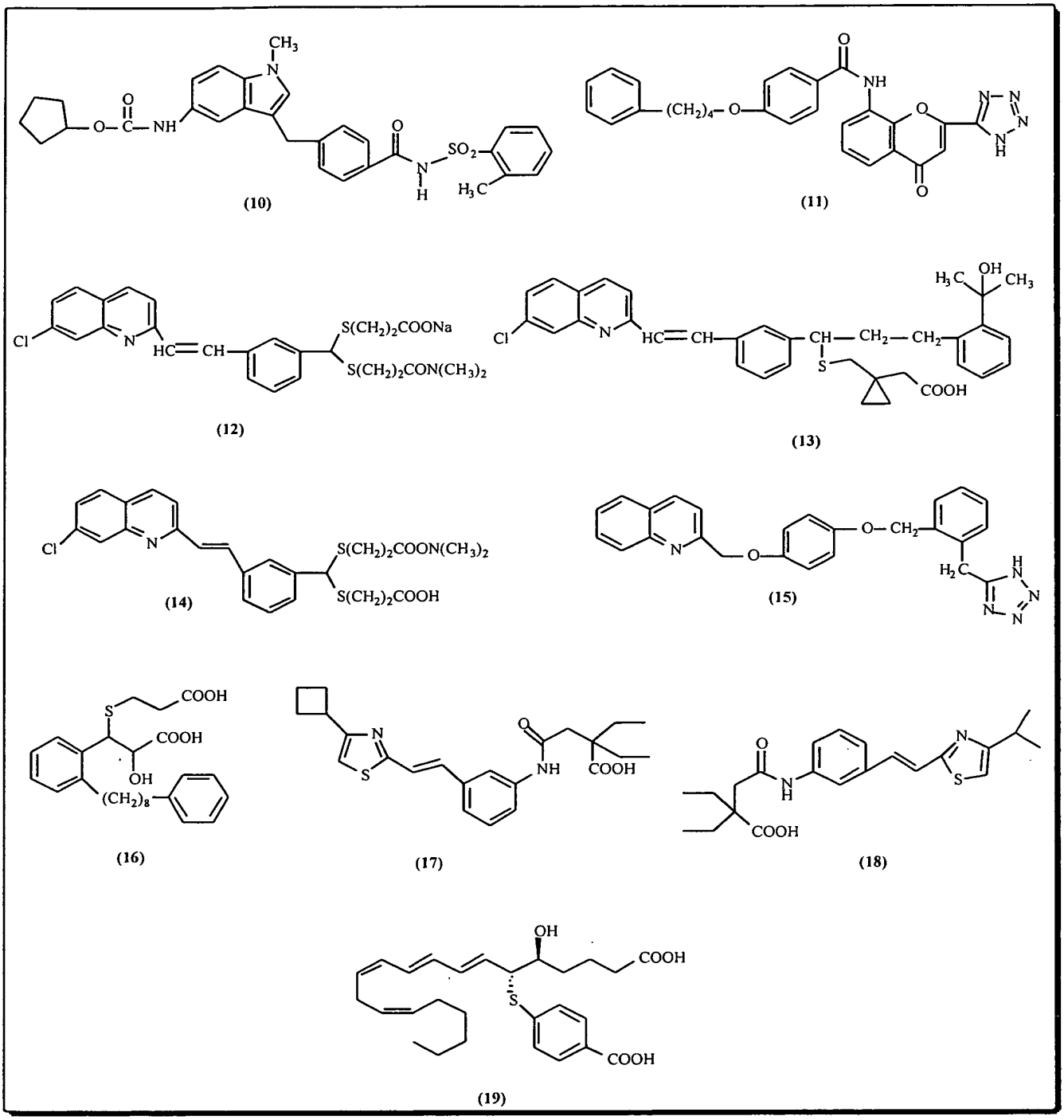
16638 has been profiled in the allergic sheep model, and shares with many other peptide LT antagonists the ability to block early and late bronchoconstriction responses in this model [46].

Two additional structure-activity studies leading to LY 163,443 [47] and to Ro23-3544 were also reported [48]. In the first study, two LTD_4 antagonists, L-647,438 and L-649,923 were evaluated using a hyperactive rat model. After oral administration both the compounds were found to inhibit antigen-induced dyspnea with ED_{50} values of 5.75 and 1.3 mg/kg, respectively. A longer duration of action was observed for L-649,923 compared to L-647,438, but there were no significant effects on late stage allergic responses and no adverse effects like abdominal discomfort and diarrhea seen typically with this class [49].

Another study demonstrated that REV-5901 inhibited leukotriene production and antagonized their action [50]. In human lung tissues it also inhibited leukotriene and antigen-induced histamine release [51].

Second-Generation Cysteinyl Receptor Antagonists

The second-generation of cysteinyl receptor antagonists currently in development is approximately 200 times more potent than the previously reported compounds [52]. These include compounds such as ICI-204,219(**10**) (zafirlukast), ONO-1078 (**11**) (pranlukast), MK-0679 (**12**) (verlukast), MK-0476 (**13**) (montelukast), MK-571(**14**) (L-66071), RG-12525 (**15**), SKF-104,353 (**16**) (pobilukast), R024-5913 (**17**) (cinalukast), MCI-0826 (**18**), Bay u9773 (**19**), and Bay-X7195 (**20**). Zafirlukast is one of the most potent oral LTD_4 antagonists reported thus far. When this compound is administered orally or by inhalation it protected against exercise-induced asthma [53-55]. The oral activity of zafirlukast is 30 fold greater than that of tomelukast [18]. A single dose of 40 mg of this compound in asthmatic patients significantly attenuated the early and late phase brochoconstriction to inhaled allergen,both in terms of the maximum decrease in FEV1 and the area under the allergen dose-response curve.



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Pretreatment of aspirin-intolerant individuals with pranlukast, a relatively new cysteinyl leukotriene receptor antagonist, significantly inhibited inhalation challenge with dipyrone [56]. Fujimura *et al.*, conducted a double-blind, randomized, placebo-controlled, 2-phase, cross-over study of oral pranlukast in stable asthmatic patients. After one week of treatment, there was a small, but significant improvement in bronchial hyper-responsiveness [57]. Zafirlukast (as an aerosol) and pranlukast, also provided some protection in cold-air challenge studies [58]. It was also shown that another receptor antagonist, verlukast, was effective against aspirin-induced bronchoconstriction [59].

A study was conducted to investigate the effects of several of the more potent leukotriene receptor antagonists on both the immediate and delayed response to challenge.

Intravenous MK-571 was shown to reduce the early reaction to inhaled allergen by 88% and the late response by 66 % [60]. However, in a placebo-controlled trial, MK-571 attenuated exercise–induced bronchoconstriction in subjects with stable asthma [61]. Preparation and evaluation of the two enantiomers of MK-571 (S-(+)-isomer and R- (-)-isomer) revealed comparable binding in isolated human lung membranes [62]. In contrast, the (+) isomer was slightly more potent than the (-)-isomer as an antagonist of LTD_4 contractions in guinea pig trachea [63]. Comparison of the blood levels of both isomers showed that the (-) isomer gave higher levels at all time points taken and was cleared more slowly than the (+) isomer [64]. On the other hand, MK-571 given orally for 4 weeks was shown to be effective at reducing symptom scores, β_2 agonist use and spirometry [65].

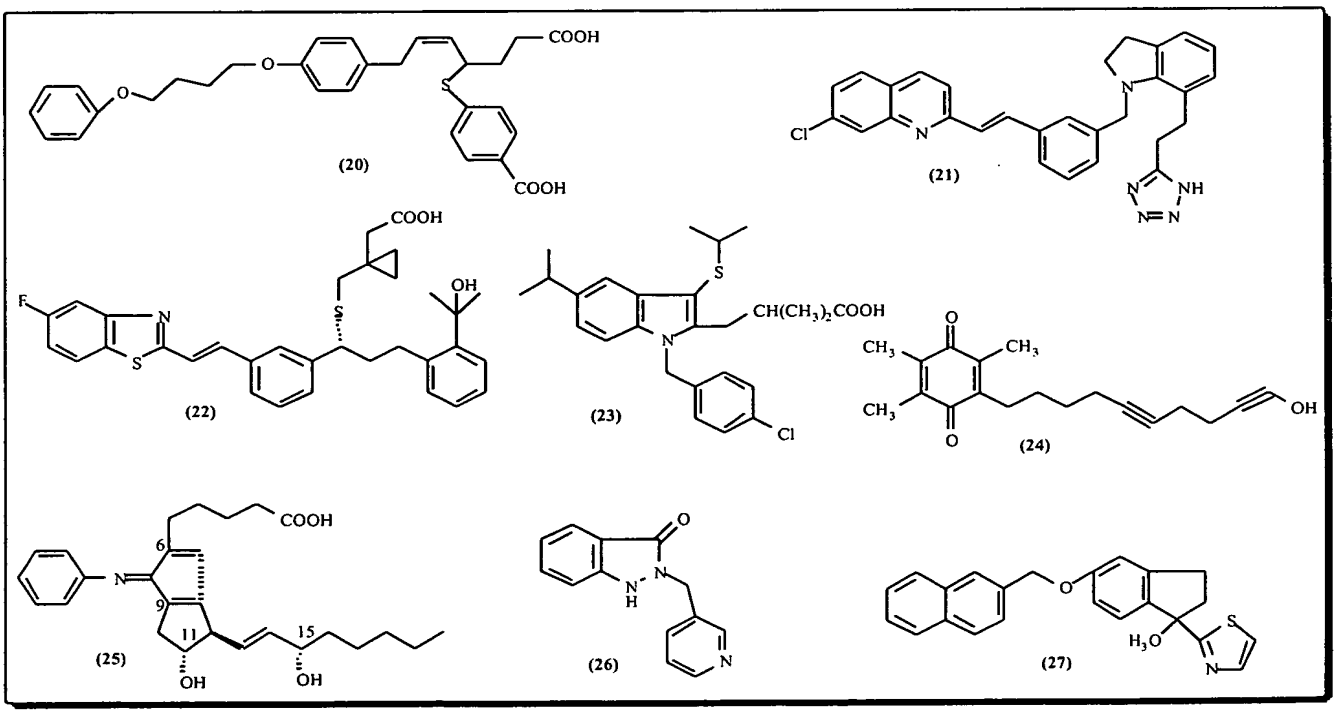
Pobilukast antagonized LTD_4-induced guinea pig trachea contraction without significant effect on the LTC_4 receptors. However, on human bronchial tissue it antagonized both LTD_4 and LTC_4 receptors [66]. In a phase II challenge study in asthmatics, zafirlukast, montekulast and cinalukast progressed to outpatient trials for varying lengths of time. It was found that, all three compounds were effective against exercise-induced asthma in small studies [54, 67, 68] and only zafirlukast and montekulast were effective in antigen-induced bronchospasm [69,70]. To date, four Cys-LT_1 antagonists have progressed to phase 3 clinical trials in outpatients, and only RG 12525 gave modest improvement in FEV 1 [71].

Zafirlukast (20 mg bid in orally) was shown to statistically increase FEV1 volumes in one 3 month trial but not in another [72, 73]. However, in both studies the symptoms and peak flow measurements were positively effected. Pranlukast has been studied in larger trials and had positive effects on FEV1 at a 450 mg BID dose [74]. However the effect tended to diminish at the end of the study. Finally, a daily dose of 10 mg of montelukast was reported to increase FEV 1 in an outpatient trial [75].

Two new antagonists described recently are the fluoro-quinoline OT 4003 **(22)**, and the quinoline substituted dihydroindole LY 302905 **(21)** [76,77]. These compounds are potent orally active antagonists of the Cys-LT_1 type. In addition Bay u9773, was shown to be selective for Cys-LT_2 receptors in guinea pig ileum preparations that contain both types of receptors [78].

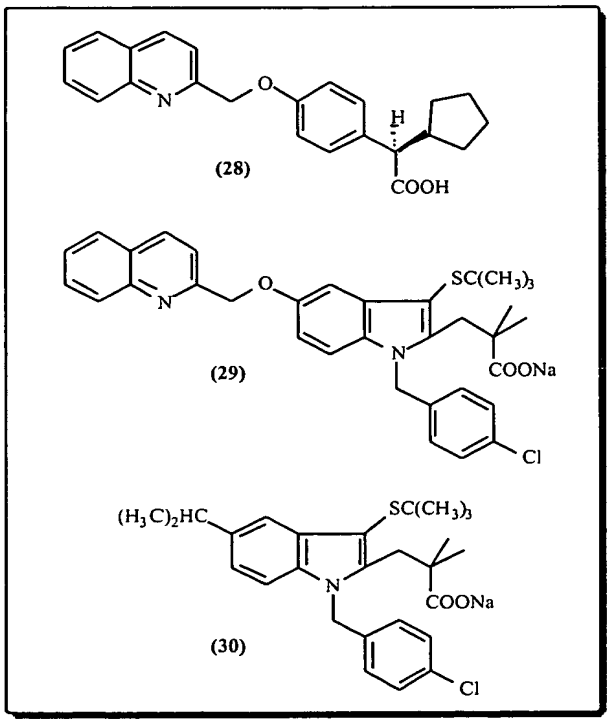
5-Lipoxygenase and FLAP Inhibitors

Leukotriene synthesis inhibitors act by either directly inhibiting 5-lipoxygenase (5-LO) or by binding to FLAP (5-lipoxygenase-activating protein) and indirectly inactivating 5-LO (Fig. 1). Clinical evaluation of 5-lipoxygenase inhibitors for the treatment of asthma has generally lagged behind that of LTD_4 receptor antagonists, due to the difficulty of finding appropriately safe and selective compounds. The 5-lipoxygenase is the first committed enzyme in the biosynthetic route of leukotrienes and inhibition of 5-LO results in the elimination of both LTB_4 and the cysteinyl leukotrienes [79]. This enzyme is also quite complex and requires many cofactors for activity. For example, it can be inhibited by trapping free radicals, chelators, iron reduction, and by binding at an active or regulatory site [80,81]. Examples of direct 5-LO inhibitors include zileuton **(23)**, docebenone (AA-861) **(24)**, piriprost (U-60257) **(25)**, ICI-207968 **(26)** and ICI-216,800 **(27)**.



As with leukotriene receptor antagonists, the initial studies with 5-lipoxygenase inhibitors were disappointing. Piriprost is an inhaled 5-LO inhibitor, but failed to have any effect on allergen or exercise-induced bronchoconstriction in asthmatic patients [82]. Another agent, docebenone produced no change in bronchial responsiveness to acetylcholine in patients with asthma [83] but was also later shown to have very poor oral bioavailability [57]. Initial results with a more recent compound, zileuton, have been more encouraging. In a double-blind, randomized study in asthmatic males, airway reactivity to hyperventilation of cold dry air was reduced, following oral administration of the drug [84]. This indicates that attenuation of the asthmatic response to cold dry air may be accomplished through selective inhibition of 5-LO. Allergen-induced nasal congestion was significantly decreased by zileuton in human volunteers, suggesting the potential therapeutic importance of 5-LO inhibition in allergic rhinitis [85]. Evaluation of the structure-activity relationships for a number of related hydroxamic acids revealed a correlation between hydrophilicity and 5-LO inhibitory potencies [86]. A series of 2-substituted indazolones were identified as potent and selective inhibitors of 5-LO [87]. The 2-pyridylmethyl analog ICI-207968 inhibited LTB_4 release from calcium ionophore stimulated rats, blood after oral dosing without inhibition of PGE_2 synthesis. ICI-216,800 exemplifies the first chemical series to show a stereospecific interaction in the inhibition of 5-LO [88].

Antagonists of the 5-lipoxygenase-activating protein such as Bay X1005 **(28)**, MK-591 **(29)**, and MK-886 (L-663,536) **(30)**, would be expected to inhibit both branches of the leukotriene pathway (like 5-LO inhibitors). Possible differences between the two types of inhibitors have been observed. The first difference is seen in systems where there is a high arachidonic acid concentration. Leukotriene effects tend to be weakly inhibited by inhibition of FLAP rather than by direct 5-LO inhibiton [89]. However, this phenomena has not been observed in animal models where physiological stimuli caused leukotriene production. Secondly, even though the FLAP protein has been shown to be structurally related to LTC_4 synthase [90], no detailed study of the relative potency of a class of FLAP inhibitors against both enzymes has been published (although a FLAP inhibitor has been shown to inhibit LTC_4 synthase).



MK-886 is an example of a compound which inhibits leukotriene synthesis by binding to FLAP. It has also been shown to inhibit LTB_4 production in human whole blood *in vitro* and causes a modest attenuation of the early and late phase asthmatic responses to inhaled allergen [12]. No adverse effects were reported in clinical trials with MK-886 [57] (one week treatment with 450 mg dose). This compound was examined in atopic subjects with documented early (EAR) and late asthmatic reactions (LAR) and in the subsequent increase in airway hyper-responsiveness to histamine [91]. When given orally, MK-886 inhibited the antigen-induced EAR and delayed the LAR, but did not afford protection against the increase in airway hyper-responsiveness.

The FLAP antagonists Bay X1005 and MK-591 were also found effective against antigen-induced bronchospasm in asthmatics [92-94].

CONCLUSION

Leukotrienes play an important role in the pathogenesis of asthma. They have the capacity to cause bronchoconstriction, increase the permeability of the vasculature and mucus secretion and induce the recruitment of inflammatory cells. This suggests that leukotrienes and the pathway contains many attractive therapeutic targets and are involved in a complex inflammatory process which leads to clinical manifestations of asthma. To date promising results are available from studies with leukotriene receptor antagonists, 5-lipoxygenase inhibitors, and 5-lipoxygenase-activating protein inhibitors. Furthermore, leukotriene receptor antagonists and synthesis inhibitors are effective in a number of laboratory models of asthma.

In summary, it appears from the data that the next generation of anti-asthmatic therapies will emerge from the current preclinical and clinical studies being conducted with leukotriene receptor antagonists, 5-lipoxygenase inhibitors or inhibitors of the 5-lipoxygenase-activating protein.

ABBREVIATIONS

FLAP = 5-Lipoxgenase-activating protein

5-HPETE = 5-Hydroperoxy-eicosatetraenoic acid

5-LO = 5-Lipoxygenase

PG = Prostaglandins

TX = Thromboxane

CO = Cyclooxygenase

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Exhibit 2

Activation of the human neutrophil 5-lipoxygenase by exogenous arachidonic acid: involvement of pertussis toxin-sensitive guanine nucleotide-binding proteins

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1 The mechanism by which incubation of human peripheral blood neutrophils with exogenous arachidonic acid leads to 5-lipoxygenase product synthesis was investigated.

2 Incubation of neutrophils with arachidonic acid caused a concentration- and time-dependent synthesis of leukotriene B_4, its Ω-oxidation products, and 5-hydroxyeicosatetraenoic acid.

3 The threshold concentration of arachidonic acid required for this effect was equal to, or greater than 3.3 μM and the synthesis increased with up to 33 μM arachidonic acid, the highest concentration used. Synthesis induced by arachidonic acid increased with time for up to 15 min and the major products detected were the Ω-oxidation products of leukotriene B_4.

4 Pre-incubation of neutrophils with pertussis toxin inhibited the synthesis of 5-lipoxygenase products induced by arachidonic acid by 75% or more, but had no effect on either arachidonic acid-induced synthesis of the 15-lipoxygenase product, 15-hydroxyeicosatetraenoic acid, or activation of the 5-lipoxygenase induced by the calcium ionophore A23187.

5 Pre-incubation of neutrophils with granulocyte-macrophage colony-stimulating factor lead to enhanced leukotriene synthesis in response to arachidonic acid.

6 These results imply that exogenous arachidonic acid is not only used as a substrate, but also activates the 5-lipoxygenase. Possible mechanisms of action are discussed.

Introduction

By virtue of their potent effects on cells of the immune system, the 5-lipoxygenase metabolites of arachidonic acid are believed to play important roles in inflammation and allergy (Sirois & Borgeat, 1984; Lewis & Austen, 1984). In particular, leukotriene B_4 is a stereospecific activator of neutrophil chemotaxis (Ford-Hutchinson *et al.*, 1980; Malmsten *et al.*, 1980), calcium mobilization (Naccache *et al.*, 1981; White *et al.*, 1983), aggregation (Ford-Hutchinson *et al.*, 1980) and degranulation (Hafstrom *et al.*, 1981). The actions of leukotriene B_4 are receptor-dependent (Goldman & Goetzl, 1984) and are blocked by the guanine nucleotide-binding protein (G-protein) inhibitor, pertussis toxin (Becker *et al.*, 1985).

For 5-lipoxygenase product generation to occur, both release of endogenous arachidonic acid and activation of the 5-lipoxygenase are required. These two events are calcium-dependent (Feinstein & Sha'afi, 1983; Borgeat *et al.*, 1983; Samuelsson *et al.*, 1987). Furthermore, it has recently been demonstrated that activation of the 5-lipoxygenase may require its calcium-dependent translocation to the cell membrane (Rouzer & Samuelsson, 1987; Rouzer & Kargman, 1988). Therefore, although the requirement of calcium for release of endogenous substrate may be overcome by supplying exogenous substrate, it is likely that intracellular calcium mobilization is still necessary for significant activation of the 5-lipoxygenase.

Recently, there has been considerable focus on the action of arachidonic acid on neutrophils. At high concentrations (50–100 μM), arachidonic acid stimulates translocation of protein kinase C (McPhail *et al.*, 1984; Cox *et al.*, 1987). At lower concentrations (0.1–50 μM), incubation of neutrophils with arachidonic acid results in mobilization of intracellular

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calcium (Smith *et al.*, 1987; Sha'afi *et al.*, 1980), aggregation (O'Flaherty *et al.*, 1979), production of superoxide (Maridonneau-Parini & Tauber, 1986; Badwey *et al.*, 1987; Clark *et al.*, 1987), and degranulation (Smith *et al.*, 1987; O'Flaherty *et al.*, 1979). Although the exogenous concentrations of arachidonic acid required to activate these cells are considerably higher than those for other compounds such as Paf, fMet-Leu-Phe and leukotriene B_4, they may be achieved in inflamed tissue (Hammarstrom *et al.*, 1975; Greaves, 1986).

In light of these previous reports which have characterized arachidonic acid as a neutrophil stimulus, we investigated and characterized the effect of exogenous arachidonic acid on the 5-lipoxygenase. The results indicate that the interaction of exogenous arachidonic acid with the human neutrophil 5-lipoxygenase system involves not only the presentation of substrate, but also activation of the 5-lipoxygenase enzyme that is mediated, at least in part, via pertussis toxin-sensitive G-proteins.

Methods

Cell separation

Whole blood was obtained by venepuncture, and following dextran sedimentation of erythrocytes, neutrophils were purified by centrifugation on Ficoll-Paque cushions (Boyum, 1968). The erythrocytes remaining in the final pellet were removed by hypotonic lysis with water (20 s), and the cells were resuspended at a final concentration of 10^7 cells ml^{-1}. The entire cell separation was carried out at room temperature. The percentage of neutrophils in the cell suspensions used in this study exceeded 97%, and cell viability as determined by trypan blue exclusion was greater than 98%.

Cell incubations

The cell suspensions were warmed to 37°C for 5 min before incubation with arachidonic acid. Arachidonic acid, A23187 and 15-HPETE were dissolved in dimethylsulphoxide (DMSO) at a 1000 fold higher concentration than was finally required. The final concentration of DMSO present (maximum of 0.2%) did not stimulate detectable leukotriene synthesis. After the desired incubation time with the stimuli, the cells were inactivated by the addition of 500 μl of an ice-cold mixture of methanol/acetonitrile (50/50, v/v), containing internal standards (25 ng ml^{-1} of prostaglandin B_2 and 19-OH prostaglandin B_2). The samples were then diluted by the addition of 500 μl of distilled water, and stored at −20°C until analysed by r.p.-h.p.l.c.

In the experiments involving pertussis toxin, the cell suspensions were pre-incubated with the toxin at a final concentration of 0.25 μg ml^{-1} for 2 h at 37°C. During this period of time, the cells were gently swirled in a rotary water bath (New Brunswick Scientific, Edison, New Jersey, U.S.A.). After 2 h, the cells were incubated under the desired conditions, inactivated and stored as described above prior to r.p.-h.p.l.c. analysis.

To examine the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) on 5-lipoxygenase product synthesis in response to arachidonic acid, neutrophils were pre-incubated with 200 pM GM-CSF at room temperature for 1 h. We have previously shown that these incubation conditions are optimal for maximal enhancement of leukotriene synthesis in response to Paf, fMet-Leu-Phe and A23187 (DiPersio *et al.*, 1988). The cells were then warmed to 37°C for 5 min before stimulation with arachidonic acid.

Reverse phase-h.p.l.c. analysis of lipoxygenase products

Analysis of lipoxygenase products was carried out by r.p.-h.p.l.c. as described previously, using an on-line extraction procedure (Borgeat & Picard, 1988). Briefly, the denatured cell suspensions were centrifuged at 600 *g* for 10 min to remove precipitated material, and the supernatants were injected directly onto a Nova-Pak C_{18} (5 × 100 mm, 4 μm particles, Waters Millipore) protected with a Guard-Pak (μBondaPak C_{18}) cartridge (Waters Millipore). The various lipoxygenase products were eluted at 1.5 ml min^{-1} with gradients of organic solvents. Elution was monitored with fixed wavelength u.v. photometers at 229 and 280 nm. The compounds were identified by comparison of their retention times with those of authentic standards and by the ratio of their u.v. absorbances at 229 and 280 nm. 5,15 diHETE was measured at 229 nm under the same assay conditions as described above. Quantitation was performed by comparing peak heights of calibrated standards and corresponding compounds in samples after correction for recovery using the internal standard PGB_2. The lower level of detection was 0.5–1 ng.

Chemicals

Calcium ionophore A23187 and 5, 8, 11, 14 (all-*cis*) eicosatetraenoic acid (arachidonic acid) were obtained from the Sigma Chemical Company (St. Louis, Missouri, U.S.A.). Hank's Balanced Salt Solu-

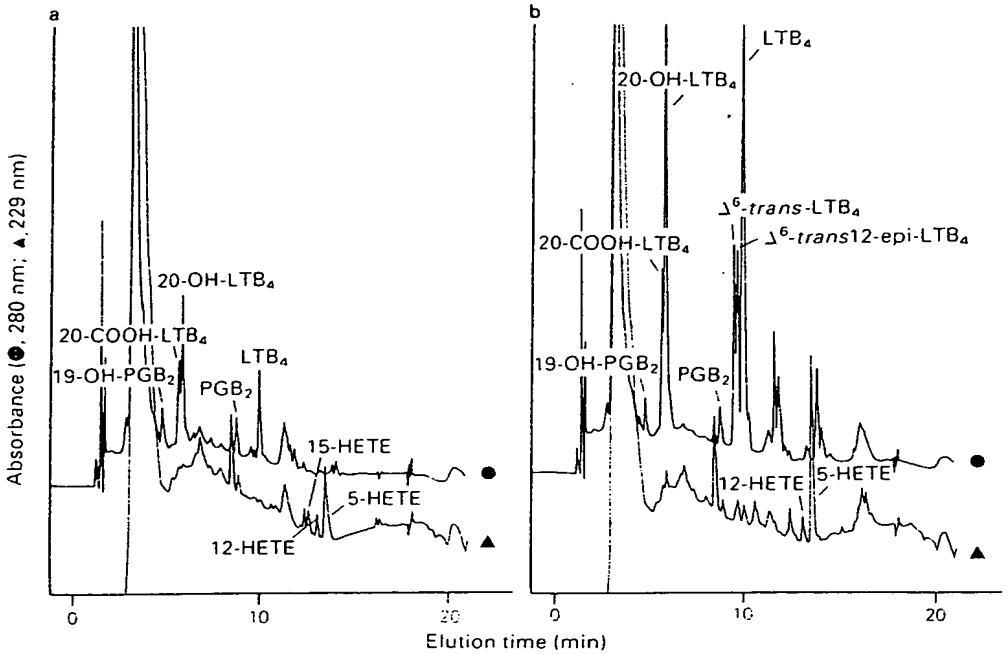

Figure 1 Reverse-phase h.p.l.c. chromatograms of lipoxygenase products from human neutrophils. Neutrophils from healthy human donors were prepared as described (see Methods), resuspended at a final concentration of 10^7 cells ml^{-1}, and incubated at 37°C for 15 min under the following conditions: (a) addition of 33 μM arachidonic acid, or (b) addition of 1.0 μM calcium ionophore A23187. After 15 min, the reactions were stopped by the addition of 500 μl of an ice-cold mixture of methanol/acetonitrile containing 12.5 ng of the internal standards (PGB_2 and 19-OH-PGB_2), and diluted to approximately 2 ml with 500 μl of distilled water. After centrifugation, the entire supernatant was injected. The amounts of PGB_2 and 19-OH-PGB_2 present in each chromatogram are therefore 12.5 ng. Elution, detection and quantitation were achieved as described (see Methods). Attenuation settings were 0.02 and 0.05 a.u.f.s. for 280 nm and 229 nm respectively. These results are from one experiment that is representative of at least ten others.

tion (HBSS) was from GIBCO (Burlington, Ontario, Canada), Ficoll-Paque was purchased from Pharmacia (Dorval, Québec, Canada) and all solvents were h.p.l.c. grade from Anachemia (Montréal, Canada). Granulocyte-macrophage colony-stimulating factor (GM-CSF) was a generous gift from the Genetics Institute (Cambridge, Massachussetts, U.S.A.), fura-2 was purchased from Molecular Probes Inc. (Eugene, Oregon, U.S.A.). 15S-hydroperoxy-eicosatetraenoic acid (15-HPETE) was synthesized from soy-bean lipoxygenase as previously described (Gardner, 1975), using arachidonic acid as substrate. The fatty acids (arachidonic acid and 15-HPETE) were purified either by silicic acid chromatography or reverse-phase high performance liquid chromatography (r.p.-h.p.l.c.) prior to use. Pertussis toxin was purchased from List Biological Labs Incorporated (Campbell, California, U.S.A.).

Results

Reverse phase-h.p.l.c. lipoxygenase products from arachidonic acid-stimulated human neutrophils

When human peripheral blood neutrophils were exposed to arachidonic acid they produced leukotriene B_4, its Ω-oxidation products (20-OH and 20-COOH-leukotriene B_4) and 5-HETE. Occasionally, we also observed synthesis of the non-enzymatically derived all-*trans* isomers of leukotriene B_4. However, this was not a consistent response during the course of this study. Figure 1a shows the characteristic arachidonic acid metabolite profile when the cell suspensions were exposed to 33 μM arachidonic acid for 15 min. Under these conditions, the major product was leukotriene B_4 (taking into account the combined synthesis of leukotriene B_4 and its Ω-

oxidation products). Production of the 15-lipoxygenase metabolite, 15-HETE was also observed under these conditions as was 12-HETE, which was derived from contaminating platelets. The 5-lipoxygenase product synthesis induced by arachidonic acid was not as powerful as that observed with the calcium ionophore A23187 (Figure 1b), where larger quantities of leukotriene B_4 and its metabolites as well as 5-HETE were detected (no 15-HETE was observed when A23187 was used as a stimulus). In addition to these compounds, A23187 also induced the production of the non-enzymatically-derived diHETEs.

Concentration-dependence of the effect of arachidonic acid on 5-lipoxygenase product synthesis

To characterize further the effect of exogenous arachidonic acid on 5-lipoxygenase product synthesis, neutrophils were incubated with increasing concentrations of the fatty acid for 15 min (Figure 2). A concentration-dependence was observed, with maximal formation of 5-lipoxygenase products at the highest dose of arachidonic acid used (33 μM). The threshold in all experiments was between 3.3 μM and 10 μM, depending on the donor. The major products were 20-OH- and 20-COOH-leukotriene B_4. The synthesis of the 15-lipoxygenase product, 15-HETE, also increased with increasing concentrations of arachidonic acid. Maximal stimulation of leukotriene synthesis by arachidonic acid was observed after an incubation time of 15 min. At this time point, the product profile was characterized by a marked increase in the levels of 20-OH and 20-COOH-leukotriene B_4 while levels of 5-HETE, 15-HETE and leukotriene B_4 had plateaued (data not shown).

Effect of pre-incubation of neutrophils with pertussis toxin on the 5-lipoxygenase product synthesis induced by arachidonic acid

To examine the hypothesis that arachidonic acid not only acts as a substrate but also stimulates 5-lipoxygenase product synthesis via a pertussis toxin-sensitive G-protein, neutrophils were preincubated with 0.25 μg ml^{-1} of pertussis toxin for 2 h at 37°C before stimulation. These incubation conditions with pertussis toxin have been previously shown to cause significant inhibition of G-protein function in other systems (Becker *et al.*, 1985; Bradford & Rubin, 1985). These experimental conditions were chosen in order to maximize cell viability and experimental reproducibility. Pre-incubation of neutrophils with pertussis toxin prior to stimulation with arachidonic acid significantly inhibited 5-lipoxygenase product synthesis (Figure 3). The inhibitory effect of the toxin was observed even at the highest dose of arachidonic

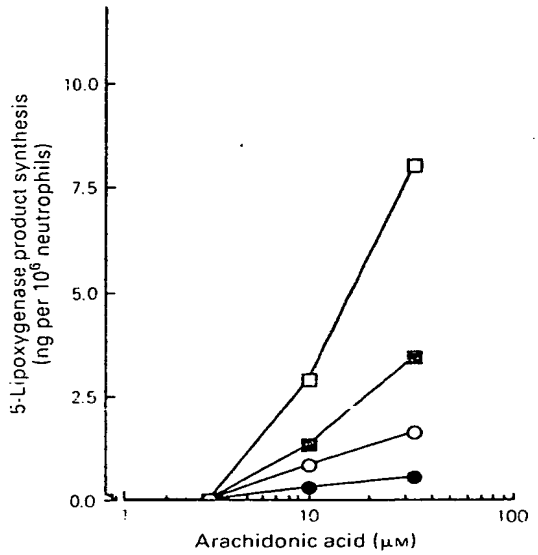

Figure 2 Concentration-dependence of the transformation of arachidonic acid by human neutrophils into 5-lipoxygenase products. Neutrophils (10^7 cells per ml) were stimulated at 37°C for 15 min with increasing concentrations of arachidonic acid. The reactions were then stopped and the resultant 5-lipoxygenase product synthesis was determined by r.p.-h.p.l.c. as described (see Methods): (●) leukotriene B_4; (■) 5-HETE; (○) 15-HETE; (□) Σ(20-OH- and 20-COOH-leukotriene B_4). The values are expressed as the mean of triplicate determinations from a single experiment which is representative of five others. Standard deviations of the mean were less than 5% of the mean.

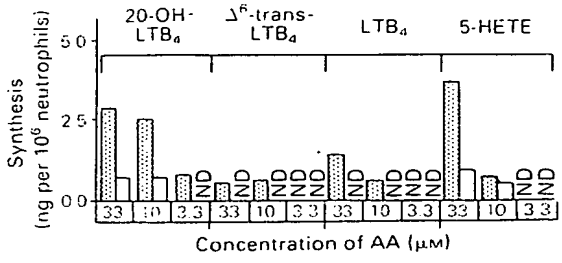

Figure 3 Effect of pre-treatment of human neutrophils with pertussis toxin (PT) on the 5-lipoxygenase product synthesis induced by arachidonic acid (AA). Neutrophils were pre-incubated for 2 h at 37°C with either 0.25 μg ml^{-1} PT or an equivalent volume of diluent control, prior to stimulation for 15 min with one of 3 different concentrations of arachidonic acid. The results are expressed as described in the legend for Figure 2. ND = not detectable. Open column with PT; stippled column without PT.

acid used (33 μM), where production of leukotriene B_4 and its all-*trans* isomers was not detected in the cells pre-treated with pertussis toxin. Furthermore, levels of Ω-oxidation products of leukotriene B_4 and 5-HETE were reduced to less than 30% of control. The same trend was observed when 10 μM arachidonic acid was used as a stimulus, and pre-treatment of the cells with pertussis toxin completely inhibited the response to 3.3 μM arachidonic acid.

In support of a specific effect of pertussis toxin on 5-lipoxygenase product synthesis, pre-incubation of neutrophils with pertussis toxin had no effect on the arachidonic acid-induced synthesis of the 15-lipoxygenase product 15-HETE while the synthesis of 5-lipoxygenase products was inhibited by more than 60% under the same conditions (data not shown).

Effect of pre-incubation of human neutrophils with pertussis toxin on the transformation of 15-HPETE into 5,15 diHETE induced by A23187 or arachidonic acid

In order to determine whether the toxin was blocking the activation pathway of the 5-lipoxygenase or directly inhibiting the enzyme, we studied its effect on the activation of the 5-lipoxygenase induced by the calcium ionophore A23187, a non receptor-dependent agonist. In these experiments, 5-lipoxygenase activity was assessed by the measurement of the formation of 5,15 diHETE from exogenous 15-HPETE, a compound previously shown to be an excellent substrate for the 5-lipoxygenase in A23187-stimulated neutrophils (Borgeat *et al.*, 1983). By using an exogenous substrate, the possibility that pertussis toxin reduced 5-lipoxygenase product synthesis via an effect at the level of substrate release was eliminated. Neutrophils were pre-incubated with or without pertussis toxin under the conditions described above, and then incubated with both 10 nM A23187 and 3.3 μM 15-HPETE for 15 min at 37°C (Figure 4). Similar experiments were carried out on neutrophils incubated with both 3.3 μM arachidonic acid and 3.3 μM 15-HPETE. At the concentrations used, neither A23187 nor arachidonic acid stimulated detectable synthesis of 5,15 diHETE when added alone (data not shown). Incubation of neutrophils with 15-HPETE alone resulted in the formation of small amounts of 5,15 diHETE (Figure 4), a response that was unaffected by pre-treatment with pertussis toxin. In combination with 15-HPETE, both arachidonic acid and A23187 induced a significant increase in the conversion of 15-HPETE to 5,15 diHETE, indicating that both compounds stimulate 5-lipoxygenase mediated metabolism of 15-HPETE. Pre-incubation of neutrophils with pertussis toxin had no significant

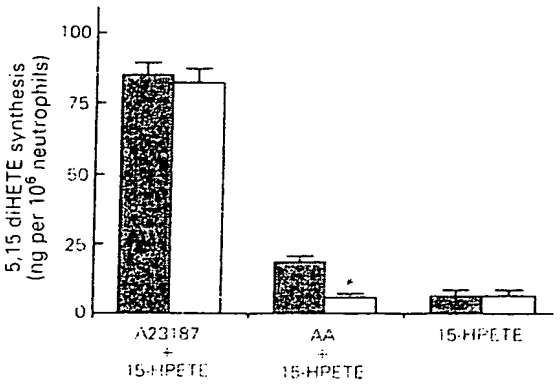

Figure 4 Effect of pre-treatment of human neutrophils with pertussis toxin (PT) on the activation of the 5-lipoxygenase by the calcium ionophore A23187 or arachidonic acid. Neutrophils were pre-incubated for 2 h at 37°C with 0.25 μg ml^{-1} PT (open columns) or the equivalent volume of diluent control (solid columns). The cells were then incubated under the following conditions: 3.3 μM 15-HPETE and 10 nM A23187, 3.3 μM 15-HPETE and 3.3 μM arachidonic acid (AA), or 3.3 μM 15-HPETE alone. After 15 min, the reactions were stopped and the resulting synthesis of 5,15 diHETE measured by r.p.-h.p.l.c. as described (see Methods). Synthesis of 5,15 diHETE under stimulation by either 10 nM A23187 or 3.3 μM arachidonic acid alone was not detectable. The results are expressed as mean of triplicate determinations of a single experiment that is representative of three; vertical bars show s.d. Significantly different from control values at $^*P < 0.01$ (Student's *t* test, $n = 3$).

effect on the synthesis of 5,15 diHETE induced by A23187 while under the same conditions, the synthesis of 5,15 diHETE induced by arachidonic acid was completely inhibited.

Effect of pre-incubation of neutrophils with GM-CSF on leukotriene synthesis in response to arachidonic acid

Neutrophils (5×10^6 ml^{-1}) were pre-incubated with GM-CSF (200 pM) for 1 h at room temperature before stimulation with increasing concentrations of arachidonic acid for either 5 or 15 min. The resulting synthesis of leukotriene B_4 and its Ω-oxidation products are shown in Figures 5a and b respectively. As shown in the previous results, increased leukotriene synthesis was observed with increasing concentrations of arachidonic acid. In these experiments, the lowest concentration of arachidonic acid with which we could detect leukotriene synthesis was 10 μM. However, when the cells were pre-incubated with GM-CSF, leukotriene synthesis was consistently detected when the cells were incubated with 3.3 μM

and sometimes as little as 1.0 μM arachidonic acid (data not shown). Furthermore, pre-treatment of the cells with GM-CSF lead to enhancement (between two and ten fold depending on the concentration of exogenous arachidonic acid) of leukotriene synthesis in response to concentrations of arachidonic acid which induced leukotriene synthesis in the cells untreated with GM-CSF.

Discussion

It has previously been shown that incubation of several different cell types with exogenous arachidonic acid results in the formation of 12- and 15-lipoxygenase products (Jorg *et al.*, 1982; Goldyne *et al.*, 1984; Lagarde *et al.*, 1984). In the case of 5-lipoxygenase products, however, the situation is different, and it is generally accepted that leukotriene synthesis in neutrophils (the system most extensively studied) requires activation of the enzyme (Borgeat *et al.*, 1983). The calcium ionophore A23187 has been shown to be a strong stimulus of the neutrophil 5-lipoxygenase (Borgeat & Samuelsson, 1979a), whereas inflammatory stimuli such as Paf and fMet-Leu-Phe activate the enzyme to a lesser degree (Salari *et al.*, 1985; Borgeat *et al.*, 1988). It is also recognized that exogenous arachidonic acid induces a small but consistent synthesis of 5-lipoxygenase products in neutrophils (Borgeat & Samuelsson, 1979b). In the present work, through the use of pertussis toxin, we have clearly demonstrated that in addition to being used as substrate, exogenous arachidonic acid also activates the human neutrophil 5-lipoxygenase, thereby reinforcing the concept that 5-lipoxygenase product synthesis requires activation of the 5-lipoxygenase.

Our studies with the G-protein inhibitor, pertussis toxin, provide strong evidence for receptor-dependent activation of the 5-lipoxygenase by exogenous arachidonic acid. G-proteins have been identified as early elements in the excitation response sequence of many cell types, and have been invariably associated with the transduction of receptor occupancy into cellular responses (Gilman, 1984). To date, the G-proteins represent the earliest known molecular components interacting with cell surface receptors (Gilman, 1984). The signalling transduction pathway in human neutrophils contains a G-protein which is linked to the cell surface receptors for many neutrophil agonists including leukotriene B_4 (Becker *et al.*, 1985), Paf (Naccache *et al.*; 1986) and fMet-Leu-Phe (Lad *et al.*, 1985), and the association between this G-protein and these receptors is specifically inhibited by pertussis toxin (Becker *et al.*, 1985; Lad *et al.*, 1985; Naccache *et al.*, 1986).

In the present work, the inhibitory effects of the toxin were specifically directed towards the stimulation of 5-lipoxygenase product synthesis by arachidonic acid, since we did not observe inhibition of the synthesis of the 15-lipoxygenase product 15-HETE. In addition, using the calcium ionophore A23187 as a 5-lipoxygenase stimulus, we were able to determine that pertussis toxin did not directly inhibit the 5-lipoxygenase enzyme. In this assay, we used the hydroperoxy fatty acid 15-HPETE as a substrate and measured its conversion into 5,15 diHETE as an indication of 5-lipoxygenase activation (Borgeat *et al.*, 1983). 15-HPETE was metabolized by neutrophils to 5,15 diHETE only to a minor extent unless A23187 or arachidonic acid were also added (Figure 5). Pertussis toxin had no effect on the ability of A23187 to activate the 5-lipoxygenase and stimulate

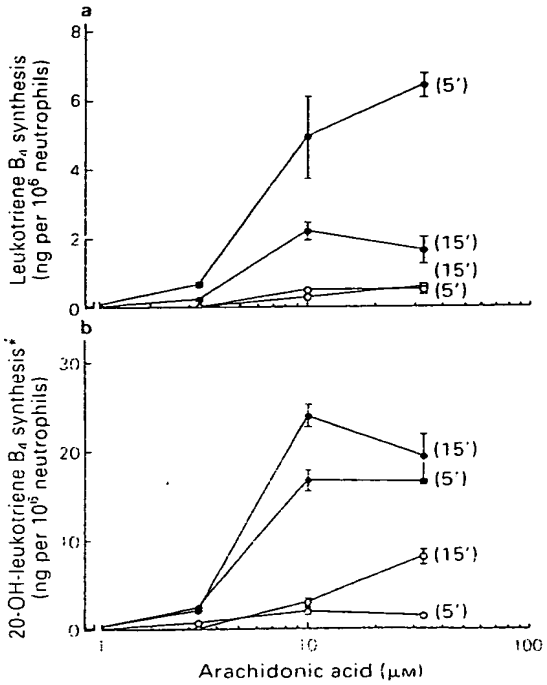

Figure 5 The effect of pre-incubation of neutrophils with GM-CSF on the synthesis of 5-lipoxygenase products induced by exogenous arachidonic acid. Neutrophils were pre-treated with 200 pM GM-CSF for 1 h at room temperature prior to stimulation with increasing concentrations of arachidonic acid for either 5 or 15 min: (●) plus GM-CSF; (○) without GM-CSF. The resultant synthesis of leukotriene B_4 and 20-OH-leukotriene B_4^* (i.e. Σ20-OH- and 20-COOH-leukotriene B_4) are shown in (a) and (b) respectively. The results are expressed as mean of triplicate determinations from one experiment that is representative of four; vertical bars show s.d.

the conversion of 15-HPETE into 5,15 diHETE, indicating that it does not directly inhibit the 5-lipoxygenase or interfere with activation of the 5-lipoxygenase by A23187. It is also important to note that arachidonic acid stimulated the transformation of 15-HPETE into 5,15 diHETE, and furthermore, that this transformation was inhibited by pertussis toxin. These results confirm our previous observation that pertussis toxin inhibits arachidonic acid-induced leukotriene synthesis and further supports our hypothesis that arachidonic acid activates the 5-lipoxygenase via a G-protein-dependent mechanism. Our results demonstrating that pre-incubation of neutrophils with GM-CSF enhanced the synthesis of leukotrienes in response to arachidonic acid also provide support to our hypothesis that arachidonic acid stimulates the 5-lipoxygenase, since we and others have recently shown that pre-incubation of neutrophils with GM-CSF enhances the ability of several other neutrophil agonists such as fMet-Leu-Phe (DiPersio *et al.*, 1988), C5a (Dahinden *et al.*, 1988) and Paf to stimulate leukotriene synthesis.

In the present study, it has proved difficult to establish the mechanism(s) involved in the activation of the 5-lipoxygenase and the stimulation of leukotriene synthesis by arachidonic acid. However, considering the established role of calcium in activation of the 5-lipoxygenase, our recent investigations on the effects of arachidonic acid on the mobilization of intracellular calcium, and the observation that leukotriene B_4 can also activate the 5-lipoxygenase, it is possible that activation of the 5-lipoxygenase by arachidonic acid is related to its ability to promote calcium mobilization, and involves activation of the leukotriene B_4 receptor, probably by a positive feedback loop due to *de novo* synthesis of leukotriene B_4 (Figure 6). The latter mechanism could be due to transformation of exogenous arachidonic acid into leukotriene B_4 by 5-lipoxygenase which is already active, or by a direct effect of arachidonic acid on intracellular calcium stores (Beaumier *et al.*, 1987), resulting in the activation of the 5-lipoxygenase, leukotriene B_4 synthesis from exogenous arachidonic acid, and subsequent activation of the leukotriene B_4 receptor. In both cases, stimulation of the leukotriene B_4 receptor would enhance the activation of the 5-lipoxygenase and consequently, leukotriene synthesis from exogenous arachidonic acid. In a separate study, we have obtained evidence that both of these mechanisms exist and account for the effect of arachidonic acid on calcium mobilization. Additional support for this hypothesis arises from the fact that the characteristics of arachidonic acid and leukotriene B_4 as neutrophil agonists are strikingly similar. Indeed, stimulation of human neutrophils by leukotriene B_4 elicits a similar range of functions as those previously described for arachidonic acid

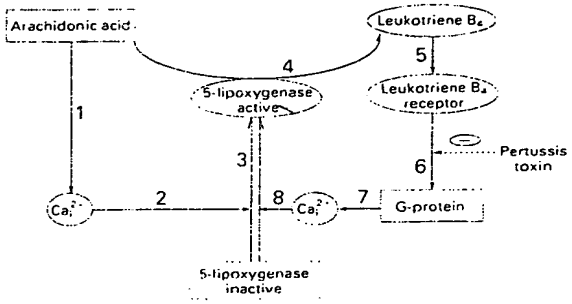

Figure 6 Possible mechanism for the stimulation of the 5-lipoxygenase by exogenous arachidonic acid. Numbers represent the postulated sequence of events. This scheme implies a critical role for calcium in the activation of the 5-lipoxygenase, and also that leukotriene B_4 activates this enzyme. In this scheme, the triggering event for the induction of 5-lipoxygenase product synthesis by arachidonic acid would be the direct effect of arachidonic acid on intracellular levels of calcium, which in turn would lead to the activation of the 5-lipoxygenase (events 1 to 3). Formation of leukotriene B_4 would occur, leading to a positive feedback effect of leukotriene B_4 on the activation of the 5-lipoxygenase (events 4 to 9). The positive feedback could eventually be switched off due to desensitization of the leukotriene B_4 receptor by leukotriene B_4.

(O'Flaherty *et al.*, 1979; Hafstrom *et al.*, 1981; White *et al.*, 1983; Smith *et al.*, 1987). Furthermore, and most importantly, cellular responses to both leukotriene B_4 (Becker *et al.*, 1985) and arachidonic acid are sensitive to pertussis toxin. The positive feedback loop could finally be switched off as a consequence of desensitization of the leukotriene B_4 receptor by leukotriene B_4, as has previously been reported (Sha'afi *et al.*, 1981).

The ability of arachidonic acid to activate the human neutrophil 5-lipoxygenase may be of considerable patho-physiological importance *in vivo*. The fact that micromolar concentrations of arachidonic acid are required to observe these effects *in vitro* does not diminish the importance of this finding since large quantities of arachidonic acid have been described in inflammatory lesions (Hammarstrom *et al.*, 1975; Greaves, 1986). Furthermore, in this work, we have demonstrated that exposure of neutrophils to GM-CSF *in vitro* increases the sensitivity of these cells to arachidonic acid (Figure 5). If GM-CSF has the same effect *in vivo*, it may have a dramatic influence on inflammation by increasing the activation of the 5-lipoxygenase not only in response to agents such as Paf and C5a (Dahinden *et al.*, 1988), but also arachidonic acid.

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Exhibit 3

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(54) Title: A NEW CHEMICAL ENTITY (ENDIPALENE) IN THE TREATMENT OF PSORIASIS

(57) Abstract

In the literature, a correlation between the inhibition of 5-lipooxygenase and anti-inflammatory and immunomodulatory activity sufficient to effectively treat psoriasis, is known. It seems that, in particular, the excellent results can be obtained with lonapalene (6-chloro-2,3-dimethoxynaphthalendioldiacetate). However, the clinical use of lonapalene has not been successful most likely due to the significant number of side effects. With these considerations, after various studies, we have identified a molecule which we have patented with the name of endipalene, which seems to guarantee notable therapeutic results without side effects.

DESCRIPTION

A NEW CHEMICAL ENTITY (ENDIPALENE) IN THE TREATMENT OF PSORIASIS

TECHNICAL FIELD

My invention relates to the field of molecules with two aromatic condensed rings (naphtalenic rings) characterized by a good activity against inflamma - tion through inhibitory actions in one or more pas- sages of the "fall" of the arachidonic acid.

BACKGROUND ART

From the beginning of the nineties in the medical literature lots of studies appeared about a molecu- le patented as lonapalene (drawnings, FIG 1) with a great power against inflammation of the skin. Lonapalene (6-chloro-2,3-dimethoxynaphtalendioldi- acetate) has a statistically significant reduction in the levels of material similar or identical to the chemoattractant arachidonate 5-lipooxygenase product, leukotriene B4 (Black A.K.,LAMP RD.,Malled AT.,Cunningham FM.,Hofbauer M., Greaves MW.; PHAR- MACOLOGIC AND CLINICAL EFFECTS OF LONAPALENE -RS43- 179- A 5-LIPOOXYGENASE INHIBITOR IN PSORIASIS; JOUR NAL OF INVESTIGATIVE DERMATOLOGY 95(1):50-4,1990 Jul)

A wide variety of agents have been reported as 5-li pooxygenase inhibitors. The majority of the series appear to be lipophilic reducing agents, including phenols, partially saturated.

Aromatics and compounds containing heteroatom-hetero atom bonds are the same. Many of these are not selec tive 5-LO inhibitors.

In vivo sistemic activity for many of these has been in general, disappointing, probably because of poor bioavailability caused by lipophilicity and metabo - lic instability (oxidation and conjugation of phenolic compounds). However, topically a number of agents have shown promise for skin inflammation, and best of all, lonapalene; (Batt DG.,5-LIPOXYGENASE INHIBITORS AND THEIR ANTI-INFLAMMATORY ACTIVITIES, PROGRESS IN MEDICINAL CHEMISTRY, 29:1 - 63, 1992). Unfortunately, the clinical utilisation of lonapalene wasn't succesful, probably because of the remarkable side effects. Lots of attempts have been made to find other topical nonsteroidal antipsoriatic agents ana - logues of lonapalene, without any success;(Venuti MC., Loe BE.,Jones GH., Young GM., TOPICAL NONSTEROIDAL ANTIPSORIATIC AGENTS.2.2,3-ALKYLIDENEDIOXYNAPHTALENE ANALOGUES OF LONAPALENE, JOURNAL OF MEDICINAL CHEMISTRY, 31(11):2132-6, 1988 Nov.). (Jones GH., Venuti MC., YOUNG JM., Murthy DV., Loe BE.,Simpson RA.,Berks AH., Spires DA.,Malonej PJ.,Kruseman M., et al., TOPICAL NON STEROIDAL ANTIPSORIATIC AGENTS .1.1,2,3,4,-TETRA-OXYGENATED NAPHTALENE DERIVATIVES, JOURNAL OF MEDICINAL CHEMISTRY, 29(8):1504-11, 1986 Aug.).

DISCLOSURE OF INVENTION

Considering what I reported before, my attention turned to find out a molecule as efficacious as lonapalene avoiding the negative side effects.

After a first screening which led me to select molecules without halogens, a molecule has been found:

2,6 or 2,7 -dimethoxynaphtalene that I patented with the name of ENDIPALENE.

Endipalene seems to have the same action mechanism of lonapalene excluding remarkable side effects.

BRIEF DESCRIPTION OF DRAWNINGS

The enclosed drawnings show the structural formulas of lonapalene (FIG. 1) and endipalene (FIG.2).

BEST MODE FOR CARRYING OUT THE INVENTION

I think that endipalene should be used topically, with a concentration between 1% or 2%. We need to continue studying in order to confirm the selective action mechanism (inhibitor of 5-LO), to check skin absorption, pharmacokinetics, metabolism. We should also confirm the absence of side effects and verify the results after long time.

INDUSTRIAL APPLICABILITY

If one considers that psoriasis afflicts 2% of the population in North Europe and an equal portion of the caucasians in the USA and that more than 91% of patients with psoriasis have a relative or first or second degree afflicted with the same disease, it is evident that there is a significant possibility to get success in the field of, if research confirm the positive results.

CLAIMS

Endipalene is an absolute novelty in the treatment of psoriasis and inflammatory skin diseases.
Compounds called naphtalenes, consisting of mono and bicyclic oxy and oxo compounds were subjected to clinical trials in various dermatoses of viral and supposed viral aetiology.
Analogues agents or derivatives (lonapalene) have been tested in psoriasis bioassay screens.
Endipalene is a novelty in this field.
Therefore my claims are:

1)The employment, topically or in other way gived, of endipalene at any concentration and in any solvent treating inflammatory skin deseases and particulary psoriasis.

2)The employment, topically or in other way gived, at any concentration and in any solvent, of endipalene treating diseases in fields different from those illustrated in paragraph n° 1).

Eur J Clin Pharmacol (1995) 48 : 155–160

Exhibit 4

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S. R. Brandwein · L. M. Dube

Population pharmacokinetics of zileution, a selective 5-lipoxygenase inhibitor, in patients with rheumatoid arthritis

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Abstract The pharmacokinetics of zileuton, a novel selective 5-lipoxygenase inhibitor, were studied in 37 patients with rheumatoid arthritis after administration of 200 mg, 400 mg, and 600 mg zileuton for 4 weeks. Patients had 6-h pharmacokinetic evaluation of zileuton on day 14. Plasma zileuton concentrations were quantitated using HPLC. Zileuton pharmacokinetic parameters were estimated using standard noncompartmental methods. A population analysis of zileuton pharmacokinetics was also performed with the NONMEM computer program. The pharmacokinetics of zileuton in patients with rheumatoid arthritis were similar to those previously estimated in normal healthy humans. The peak concentrations and the areas under the curves during the dosing interval were dose proportional. The noncompartmental means of the CL/f, terminal-phase half-life, and V/f of zileuton were approximately 545 ml min^{-1}, 1.4 h, and 64.3 l, respectively. The estimate of population typical values of the CL/f for a 70-kg person (540 ml min^{-1}) and V/f for a 70-kg person (64.8 l) from the NONMEM analysis were in agreement with the noncompartmental estimates. Differences in body weight, but not age or gender, helped explain some of the variability in the pharmacokinetics of zileuton in patients. Therefore, there is no pharmacokinetic basis for alteration of the zileuton dose size or the dosing schedule in patients with rheumatoid arthritis.

Key words Zileuton, Rheumatoid arthritis; 5-lipoxygenase inhibitor, population pharmacokinetics

Leukotrienes, products of the 5-lipoxygenase pathway of eicosanoid metabolism, have been implicated in the pathogenesis of a number of human diseases, including rheumatoid arthritis, inflammatory bowel disease, psoriasis, asthma, adult respiratory distress syndrome (ARDS), and allergic rhinitis. Zileuton (Leutrol, Abbott-64077, *N*-(1-benzo[b]thien-2-ylethyl)-*N*-hydroxyurea) is a potent, reversible, selective 5-lipoxygenase inhibitor in vitro and in vivo with substantial anti-inflammatory activity in a number of animal models [1–3]. The drug has demonstrated promising potential in the treatment of asthma [4].

The terminal disposition half-lives of zileuton ($t_{1/2}$) in dog, monkey, mouse, and rat are 7.5, 0.3, 0.9, and 2.3 h, respectively [2]. The pharmacokinetics of zileuton were investigated in healthy human volunteers after single oral administration of 25- to 800-mg doses [3]. The results showed that zileuton was absorbed rapidly with a mean t_{max} of approximately 1-3 h. The mean apparent clearance and the mean apparent volume of distribution were dose independent. Consequently, the mean elimination half-life of zileuton ($t_{1/2}$ = 2.3 h) did not change with dose. Zileuton is primarily metabolized via 0-glucuronidation in humans [5].

In a previous 28-day open study evaluating the safety and efficacy of zileuton 800 mg b.i.d. versus placebo in 24 patients with rheumatoid arthritis, analysis indicated that zileuton-treated patients had improvements that were consistently of greater magnitude for several clinical disease activity measures than were observed with placebo [6]. In particular, there were statistical differences between the zileuton and placebo groups in the swelling joint count (a primary efficacy variable), right-hand grip strength, and overall pain index [6]. The present study is the first characterization of zileuton pharmacokinetics in patients with

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toid arthritis administered dosages of 200, 400 600 mg q.i.d.

Materials, methods and patients

Study design

This study was a randomized, double-blind, parallel, multicenter, placebo and positive-controlled study of patients with active rheumatoid arthritis, comparing the safety and efficacy of zileuton 200 mg, 400 mg, and 600 mg q.i.d., ibuprofen 600 mg q.i.d., and placebo when administered for 28 days. A subgroup of patients participated in the pharmacokinetic assessment. Drug was administered in a double-blind fashion in the form of placebo and 200-mg zileuton (lot 38–225-AR, Abbott Laboratories, Abbott Park, Ill., USA) capsules. Patients were instructed to take their study doses with food.

Patients

Males and females aged 18–75 years with active functional class II or III rheumatoid arthritis as defined in the 1987 revised criteria for the classifications of rheumatoid arthritis by the American Rheumatism Association [7] were eligible for study participation. Females were not pregnant or nursing and were postmenopausal, surgically sterile, or using adequate contraceptive measures (oral contraceptives or intrauterine device). Males were using adequate contraception as well. Patients were excluded for the following reasons: concurrent use of warfarin or theophylline; use of systemic or intra-articular corticosteroids, methotrexate, azathioprine, antimalarials, gold salts, or penicillamine within 90 days prior to participation; any history of use of cyclosporine or cyclophosphamide; history of malignancy within 5 years prior to participation; active hepatic, cardiac, renal, neurologic, endocrine, psychiatric, or haematologic disease; and hypersensitivity to any nonsteroidal antiinflammatory drug. All participants granted written, informed consent, as approved by the local institutional review board, prior to participation.

Blood sampling

Five-milliliter blood samples were collected pre-study; 3 h following dosing on study days 7, 21, and 28; and immediately prior to dosing and 1, 2, 3, 4, and 6 h following the morning dose on study day 14. Samples were centrifuged and the resultant plasma was stored at −10°C or colder until analysis.

Zileuton assay

Plasma zileuton concentrations were quantitated using a sensitive and specific reverse-phase high-performance liquid chromatographic (HPLC) technique. In brief, zileuton and internal standard were extracted from plasma samples using solid-phase extraction (BondElut, Varian, Harbor City, Calif., USA) and eluted with ether. After evaporation of the ether, the residues were reconstituted in mobile phase for HPLC analysis. Separation was accomplished on a Supelco Supelcosil LC-18 (250 × 4.6 mm ID; 5 μm particles) column at ambient temperature with ultraviolet detection at 260 nm. The mobile phase consisted of 70% H_2O: 15% CH_3CN: 5% CH_3OH: 10% tetrahydrofuran (THF), containing 40 mM NaH_2PO_4, 7.5 mM H_3PO_4 and 5.0 mM acetohydroxamic acid (NAHA). Calibration curves for zileuton were linear in the range of 0.01–10.0 $\mu g/ml^{-1}$. The lower limit of quantitation was 0.02 mg/l^{-1}. The mean correlation coefficient of the calibration curves was 0.999. Interassay precision (coefficients of variation) ranged from 3.59 to 7.74% over a concentration range of 0.02 to 0.75 mg/l^{-1}, and accuracy (difference from theoretical) ranged from −1.07 to 9.52% over the same concentration range. Interassay accuracy (percentage deviation from nominal values) during validation ranged from 1 to 7% for zileuton and interassay precision (percentage coefficients of variation, %CVs) ranged from 3 to 14%.

Pharmacokinetic/pharmacodynamic and statistical analysis

Noncompartmental analysis

The peak plasma concentrations (C_{max}), the times to the peak concentrations (peak times, t_{max}), the trough plasma concentrations prior to the morning dose ($C_{min}AM$), and the trough plasma concentrations after the morning dose ($C_{min}PM$) of zileuton were taken directly from the plasma concentration measurements. The area under the plasma concentration versus time curve of zileuton for the dosing interval after the morning dose on day 14 ($AUC_{0\text{-}6}$) was calculated by the linear trapezoidal rule. For each concentration-time curve, β, the terminal-phase elimination rate constant, was estimated by the negative of the slope of the straight line obtained by regression of the logarithms of the measurable concentrations versus time at the log-linear terminal phase of the curve. The terminal-phase elimination half-life ($t_{1/2}$) was calculated as $\ln(2)/\beta$. The total apparent oral plasma clearance (CL/f, where f is the bioavailability factor) of zileuton was calculated by dividing the administered dose by the AUC. The apparent volume of distribution (V/f, where f is the bioavailability factor) of zileuton was calculated by dividing CL/f by β.

Population analysis

A retrospective population analysis of zileuton pharmacokinetics, i.e., plasma concentrations versus time, was also performed with the NONMEM computer program (version IV, level 1.0) developed by Beal and Sheiner [8]. A one-compartmental model with first-order absorption at steady state was used to describe the plasma concentration-time profile of zileuton.

Statistical model for interindividual variability

The pharmacokinetic parameters were modeled according to a constant coefficient of variation (CCV) or proportional error model as follows:

$$p_j = \bar{p} + \bar{p} \cdot \eta_{p_j} = \bar{p} \cdot (1 + \eta_{p_j})$$

where $\bar{p}$ is the population mean, η_{p_j} is the fractional error, and $\bar{p} \cdot \eta_{p_j}$ represents the difference between the parameter of the jth individual and the population mean (interindividual variability). η_{p_j} was assumed to be a gaussian variable with mean zero and variance σ^2_η. To avoid overparameterization, the pharmacokinetic parameters were assumed to be independently distributed and therefore no covariance terms were included in the model.

The effects of age, body weight, height, race, gender, smoking status, and concurrent administration of oral contraceptives, thyroid replacement therapy or estrogen replacement therapy on the oral clearance (CL/f) and the apparent volume of distribution (V/f) of zileuton were investigated. Only the inclusion of body weight in the pharmacokinetic model significantly improved the fit of the model.

The best pharmacokinetic and error models were chosen based on the likelihood ratio test with l_r-l_f, where l_r and l_f are the values of the objective function from the NONMEM fits of the reduced and full models, respectively. This statistic is approximately

distributed chi-square (χ^2) with q degrees of freedom, where q is the number of parameters for which values are fixed in the reduced model. The test statistic l_r-l_f was compared with $\chi^2_{1-\alpha}(q)$, the 100(1-α) percentile of the χ^2 distribution, for an α-level of 0.050.

After establishing that only body weight affected CL/f and V/f of zileuton, both a full model and three reduced models were then tested. The full model included body weight as a fixed effect and was based on the apparent linear effect of body weight on CL/f and V/f:

$$\widehat{CL/f_j} = \theta_2 + \theta_5 \cdot (BW_j - 70)$$
$$\widehat{V/f_j} = \theta_1 + \theta_4 \cdot (BW_j - 70)$$

where $\widehat{CL/f_j}$ and $\widehat{V/f_j}$ are the typical population values of CL/f and V/f, respectively, of patients with a body weight equal to that of the jth individual; BW_j is the body weight for the jth individual; the intercepts θ_2 and θ_1 are the population means of CL/f or V/f for a 70-kg patient; the fixed effects parameters θ_5 and θ_4 are the slope factors for the dependency of $\widehat{CL/f_j}$ and $\widehat{V/f_j}$ on BW_j. The reduced models were obtained by setting the slope factor to zero for CL/f, V/f or both, i.e., by removing the body weight dependency. Therefore, when one parameter (θ_4 or θ_5) of the full model was fixed to zero in the reduced model, a decrease of 3.84 or more in the objective function was significant at level $P < 0.050$; when both θ_4 and θ_5 were fixed to zero, a decrease of 5.99 or more indicated a significantly better fit at level $P < 0.050$. Various initial estimates of the pharmacokinetic parameters resulted in the same final estimates of the pharmacokinetic parameters.

Statistical model for residual (intraindividual) error

The NONMEM model used for the ith concentration measurement in the jth individual is given by:

$$C_{ij}(t) = f(p_j,t_{ij}) \cdot (1 + \varepsilon_{ij})$$

where $f(p_j,t_{ij})$ is the predicted concentration as defined by the pharmacokinetic model equation, p_j are the pharmacokinetic parameters of the jth individual, and t_{ij} is the ith sampling time of the jth individual. In this equation, the second term on the right side after multiplication by $f(p_j,t_{ij})$, i.e., $f(p_j,t_{ij}) \cdot \varepsilon_{ij}$, represents the residual departure of the observed concentration from the predicted concentration, i.e., the error model. It is assumed that ε is a random gaussian variable with a mean of zero and a variance σ^2_ε. This model is a constant coefficient of variation model, i.e., proportional error model.

Statistical methods for noncompartmental and demographics data

Dose group differences in demographics were investigated by one-way analysis of variance (ANOVA) with dose as the factor in the model. A one-way analysis of covariance (ANCOVA) was performed for all the pharmacokinetic parameter estimates with body weight as the covariable. Also included as variables for the analysis were the dose normalizations of AUC, C_{max}, and the average of the C_{min}AM and C_{min}PM values. The test for parallelism was performed. Since the hypothesis of equality of slopes was not rejected in any instance, a reduced model with body weight as the covariate and regimen as the factor was used. Pairwise comparisons with the least significant difference (LSD) procedure each with an α-level of 0.050 within the above modeling framework were presented.

Dose proportionality (or linearity) was investigated after dose normalizing (dividing by dose) C_{max}, AUC, and average of C_{min}AM and C_{min}PM. First, a comparison between the extreme doses (regimens A vs C) was performed by the appropriate t-statistic within the frameworks of the ANCOVA and ANOVA. Rejection of the hypothesis of equal means for regimens A and C carries a weight of evidence towards nonlinearity. Second, linear regression analysis was performed by regressing dose-normalized C_{max}, AUC, and average C_{min} versus dose. A slope statistically significantly different from 0.0 implies nonlinearity. All tests were two-tailed and any P-value ≤ 0.050 was deemed to be statistically significant.

Results and discussion

Thirty-seven patients with rheumatoid arthritis who received zileuton had complete pharmacokinetic profiles. The demographics of these patients are summarized in Table 1. The mean (SD) age, weight and height for patients participating in the pharmacokinetics assessment who received zileuton were similar for all three dose groups.

The mean plasma concentration-time profiles of zileuton for each group after multiple q.i.d. dose administration (day 14) are plotted in Fig. 1. The mean (SD) observed noncompartmental pharmacokinetic parameters of zileuton for each zileuton dosing group are summarized in Table 2.

The plasma concentrations of zileuton peaked at 1–4 h after dosing and declined monoexponentially on day 14 after q.i.d. administration of all three dose levels of zileuton (Table 2). The mean t_{max} of zileuton was approximately 2 h and was not statistically different between the three dose groups of zileuton. The mean dose normalized C_{max} value of zileuton was similar in all dose groups and was approximately 1 μg/ml per 100 mg zileuton dose. The increase in zileuton C_{max} was linearly related to zileuton dose. Linearity was tested by regression of dose-normalized C_{max} versus dose, in which the slope coefficient was not significantly different from zero ($P = 0.222$). The t_{max} values of zileuton in this study are similar to the data from normal male healthy volunteers in which the zileuton t_{max} ranged from 1.5 to 4.5 h after administration of

Table 1 Patient demographics

Group	n	Sex	Race	Age (years)	Height (cm)	Weight (kg)
					Mean (SD) (range)	
200 mg q.i.d. (group A)	13	3M/10F	12C/1B	52 (13) 23 – 68	168 (6) 157 – 179	72.0 (18.2) 48.8 – 109.5
400 mg q.i.d. (group B)	12	2M/10F	12C	51 (16) 33 – 75	168 (7) 157 – 183	70.2 (21.7) 45.5 – 132.6
600 mg q.i.d. (group C)	12	5M/7F	9C/2B/1H	48 (10) 31 – 64	171 (12) 155 – 191	77.2 (17.3) 45.8 – 103.0

C, Caucasian; B, Black; H, Hispanic

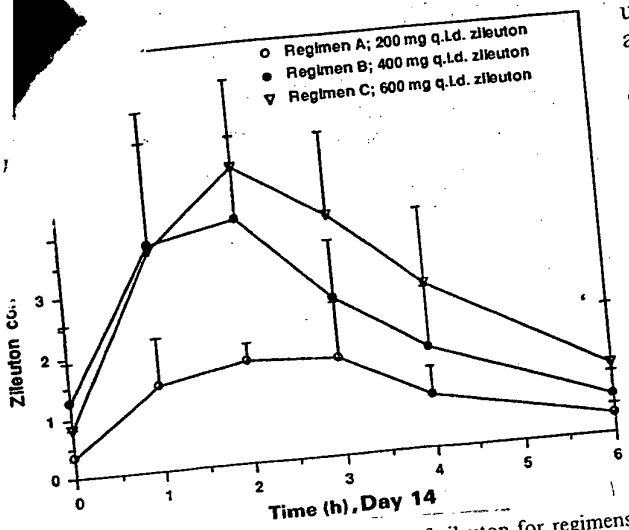

Fig. 1 Mean plasma concentrations (SD) of zileuton for regimens A, B, and C

multiple q.i.d. dosing of 600 mg zileuton to steady state (14 days).[9] The C_{max} mean of zileuton 600-mg dose in this study, 5.66 (1.41) μg/ml, is also similar to values from normal male healthy volunteers in which the mean zileuton C_{max} was 4.16 (1.12) μg/ml after administration of multiple q.i.d. dosing of 600 mg zileuton to steady state (14 days) [9].

Zileuton trough plasma concentrations prior to the morning dose (C_{min}AM), and after the morning dose (C_{min}PM) were highly variable among patients. However, C_{min}AM and C_{min}PM were not significantly different when testing the hypothesis that the differences between C_{min} AM and C_{min} PM were equal to zero at each dose level of zileuton. The results of this study are unlike the data from a normal healthy volunteer study in which the C_{min}AM was three to fourfold higher than C_{min}PM [9]. These differences are not surprising since the study in normal healthy volunteers was highly controlled, i.e., the subjects received the drug at equal intervals (q.6.h), and were in-house for the entire study and with their food, drug dosing, and activity under supervision [9]. In contrast, in the current study in rheumatoid arthritis patients, the patients were only under supervision during the morning dosing interval and the drug-dosing intervals were unequal.

After multiple q.i.d. dose administration, the dose-normalized mean areas under the plasma concentration-time curves of zileuton during the dosing interval zero to 6 h (AUC_{0-6}) were similar in all three zileuton-dosing groups, averaging approximately 3.3 μg · h/ml per 100 mg zileuton. The increase in zileuton $AUC_{(0-6)}$ was therefore linearly related to zileuton dose. The mean $AUC_{(0-6)}$ value of 18.3 (5.10) μg · h/ml in the 600 mg q.i.d. group of the present study is in excellent agreement with the mean of 17.4 (4.84) μg · h/ml obtained in normal healthy volunteers receiving 600 mg q.i.d. [9].

The mean apparent plasma clearances of zileuton in all three groups were similar and ranged from 519 to 585 ml/min. The mean CL/f of zileuton in rheumatoid arthritis patients receiving 600 mg q.i.d. was similar to the previously reported value, 619 (184) ml/min, in normal healthy volunteers who received 600 mg q.i.d. of the drug for 14 days [9]. The CL/f of zileuton was not found to be correlated to the age or affected by patient gender, but was dependent on the patient weight (Fig. 2, $P = 0.024$ in ANCOVA).

The apparent plasma clearance (CL_P/f) of zileuton is related to the apparent blood clearance (CL_B/f) of the drug by the following relationship,

$$\frac{C_B}{C_P} = \frac{CL_P/f}{CL_B/f}$$

where C_P is the concentration of zileuton in plasma and C_B is the concentration of zileuton in blood. Recently, the ratio of C_B to C_P was estimated to be

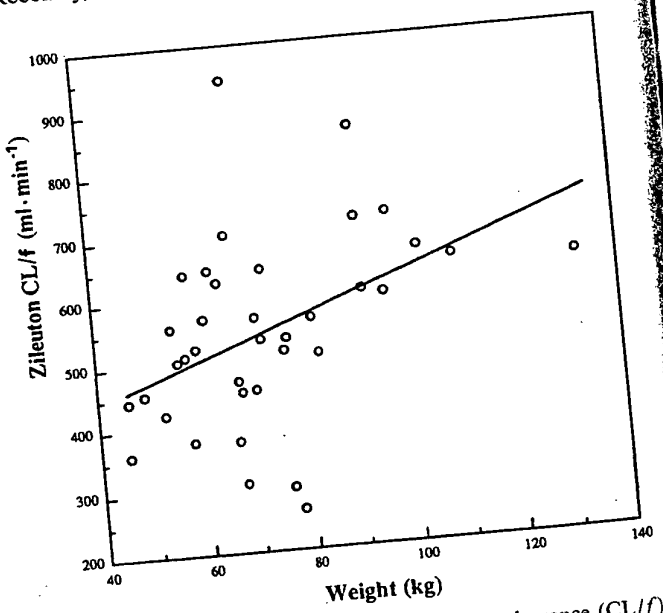

Fig. 2 Dependence of zileuton apparent plasma clearance (CL/f) on patient weight

Table 2 Noncompartmental steady-state pharmacokinetic parameters (mean ± SD) of zileuton (day 14)

	200 mg q.i.d. (group A) (N = 13)	400 mg q.i.d. (group B) (N = 12)	600 mg q.i.d. (group C) (N = 12)
t_{max} (h)	2.0 (0.8)	1.8 (0.6)	2.0 (0.9)
C_{max} (μg/ml)	2.22 (0.70)	4.60 (1.52)	5.66 (1.41)
C_{min}AM (μg/ml)	0.35 (0.45)	1.26 (1.28)	0.81 (1.11)
C_{min}PM (μg/ml)	0.33 (0.16)	0.65 (0.40)	1.17 (1.00)
AUC (μg · h/ml)	6.60 (1.92)	14.27 (4.75)	18.30 (5.10)
CL/f (ml/min)	530 (98)	519 (181)	585 (154)
$t_{1/2}$ (hr)	1.30 (0.26)	1.34 (0.33)	1.46 (0.25)
V/f (l)	59 (17)	57 (21)	77 (30)

approximately equal to 0.68 (Abbott Laboratories, internal data). Therefore, the apparent blood clearance of zileuton can be estimated to approximately equal 800 ml/min using a value of 0.68 for the C_B/C_P ratio and a value of 545 ml/min for CL_P/f. Assuming that zileuton is solely eliminated by hepatic metabolism and that the pharmacokinetics of the drug are linear, the fraction of zileuton oral dose which reaches the systemic circulation (f_L), assuming complete absorption, is given by the following equation,

$$f_L = 1 - E_L = \frac{QL}{Q_L + CL_B/f}$$

where E_L is the hepatic extraction ratio and Q_L is the total blood flow to the liver, estimated to be 1.50 l/min. Using the above equation, the maximum fraction of zileuton oral dose which reaches the systemic circulation (f_L) is estimated to be 65.2%. Thus, as much as 34.8% of an oral dose of zileuton may be eliminated by the liver during the first pass.

The mean terminal-phase half-life of zileuton was approximately 1.4 h and was similar in all three dosing groups with no significant differences. The $t_{1/2}$ of zileuton in rheumatoid arthritis patients is similar to that in normal healthy volunteers after multiple q.i.d. dosing [9].

The mean apparent volume of distribution of zileuton in the terminal elimination phase (V/f) ranged from 57 to 77 l for the three dose levels with no significant differences among the means ($P > 0.18$, ANCOVA). V/f of zileuton in rheumatoid arthritis patients was similar to values in normal healthy volunteers [9]. The mean V/f in normal volunteers was equal to 107 (21) l [9]. The V/f of zileuton in the present study was correlated to the patient weight (Fig. 3, $P < 0.001$ in ANCOVA) but not to the patient age.

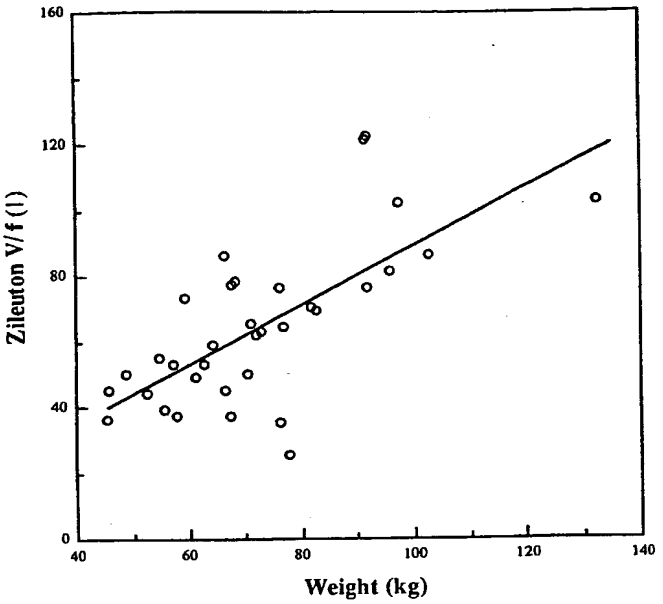

Fig. 3 Dependence of zileuton apparent volume of distribution (V/f) on patient weight

Table 3 Zileuton pharmacokinetic parameters: NONMEM estimate (%CV)[a]

	Fixed effects population means	Random effects interindividual variability[b]
k_a (h^{-1})	1.04(28.2)	43.0%
V/f (l)[c]	64.8 (16.0)	18.0%
CL/f (l/h)[c]	32.4 (3.7)	14.1%
θ_4 (l/kg)[d]	0.59 (40.0)	–
θ_5 (l/h/kg)[d]	0.172 (25.0)	–
Residual error[e]		39.2%

[a] Simultaneous fit of steady-state concentrations from 200 mg, 400 mg, and 600 mg. q.i.d. regimens;% CV are standard errors of estimation expressed as percentage of the estimates
[b] Intersubject parameter variability is the square root of the variance according to the CCV model
[c] Population estimate for a 70-kg person
[d] Population estimate of the slope factor which expresses the change in V/f and CL/f, respectively, per kilogram of body weight difference from the standard 70 kg
[e] Residual (intraindividual) variability defined as the square root of the variance according to the CCV model

Population analysis

Zileuton concentrations in plasma samples obtained as a measure of compliance were not used in the population analysis since the times of collections of these samples relative to the time of the preceding zileuton dose were uncertain. For the patients who participated in the pharmacokinetic portion of the study, the concentrations obtained on days 7, 21, and 28 were acceptable for the data analysis, as well as the data for day 14. Twelve data points from various patients were considered outliers based on preliminary data inspection and analysis. The fitted population pharmacokinetic parameters are listed in Table 3.

The NONMEM population estimates of the CL/f for a 70-kg patient (32.4 l/h or 540 ml/min) and V/f for a 70-kg patient (64.8 l) were in excellent agreement with means of these parameters using noncompartmental methods with all three groups of patients combined (545 ml/min and 64.3 l, respectively). The mean first-order rate constant and corresponding elimination-phase half-life estimated from the population analysis for a 70-kg patient were 0.50 h^{-1} and 1.39 h, respectively. The mean absorption rate constant and the corresponding half-life of absorption of zileuton were estimated to be 1.04 h^{-1} and 0.67 h, respectively.

The inclusion of body weight as covariant in the estimates of the oral clearance and volume of distribution significantly improved the pharmacokinetic model, suggesting that differences in body weight helped explain some of the variability in the pharmacokinetics of zileuton in the patients. No other

tested covariate significantly impacted the parameter estimates of the pharmacokinetic model.

The population central values of the pharmacokinetic parameters were reasonably estimated with % CV of 3.7%, 16.1%, and 28.2% for the CL/*f*, *V*/*f* and k_a, respectively (see Table 3). The estimate of interindividual variability (100* estimate of population standard deviation divided by estimate of population mean) was highest for the absorption rate constant (43.0%) and lowest for the oral clearance (14.1%). The residual variability, which includes the patient's intraindividual variability, other sources of variability such as analytical variability, and model misspecifications was estimated to be 39.2%.

Conclusions

The pharmacokinetics of zileuton in patients with rheumatoid arthritis were similar to the previously reported pharmacokinetic profile of zileuton in normal healthy volunteers after multiple q.i.d. administration of the drug. The peak plasma concentrations and the areas under the plasma concentration-time curves during the dosing interval zero to 6 h (AUC_{0-6}) of zileuton in rheumatoid arthritis patients were dose proportional after q.i.d. administration of 200 mg, 400 mg and 600 mg zileuton. The means of the apparent plasma clearance, terminal phase half-life, and apparent volume of distribution in the terminal elimination phase of zileuton were similar in all three dosing groups and were approximately 545 ml/min, 1.4 h and 64.3 l, respectively. The estimate of population typical values of the CL/*f* for a 70-kg person (540 ml/min) and *V*/*f* for a 70-kg person (64.8 l) from the NONMEM analysis were in excellent agreement with the noncompartmental estimates. Differences in body weight, but not age or gender, helped explain some of the variability in the pharmacokinetics of zileuton in rheumatoid arthritis patients. In conclusion, there is no pharmacokinetic basis for alteration of the zileuton dose size or the dosing schedule in patients with rheumatoid arthritis.

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Exhibit 5

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5-Lipoxygenase Inhibitors in the Treatment of Inflammatory Bowel Disease

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During the past decade, considerable progress has been made in elucidating the immunologic changes that occur in inflammatory bowel disease (IBD). Furthermore, advances in understanding of the immunopharmacologic mechanisms of drug action have allowed the development of new therapeutic approaches to IBD. This review briefly discusses the present knowledge of immunoregulatory changes in IBD, the biology of leukotrienes (LTs) and their relation to IBD, and the rationale for inhibiting LTs in IBD, before focusing on the evidence for potential beneficial effects of 5-lipoxygenase (5-LO) inhibitors in the treatment of IBD.

IMMUNOREGULATION IN IBD

In the pathophysiology of IBD, the mucosal immune system is involved in a sequence of events beginning with antigen processing (1). Over the last few years, several groups have presented data suggesting that class II antigen-bearing gastrointestinal epithelial cells are capable of antigen presentation to lymphocytes (2–5). If this occurs in vivo, the HLA-DR expression seen in IBD may augment the inflammatory response by increasing the amount of antigen presented to the mucosal immune system (1). There is also convincing evidence that intestinal differentiation of T cells is disturbed in IBD owing to an immunoregulatory defect in which T cells produce an abnormal cytokine profile with T_H^1/T_H^2 cell imbalance and selective proliferation of IgG-producing B cells. The precise nature of the antigens that initiate IBD is unknown, but a major focus is presently being placed on the possible etiologic role of bacterial cell wall products, including peptidoglycans, formyl-

methionyl-leucyl-phenylalanine (fMLP), and lipopolysaccharides (LPS), which nonspecifically activate macrophages, polymorphonuclear leukocytes (PMNs), and lymphocytes in the colonic mucosa. Therefore, common bacterial cell wall products and molecules capable of initiating and activating an inflammatory immune response in a genetically predisposed individual may initiate a sequence of immunologic processes that are not appropriately downregulated (1).

To summarize the sequence of events that lead to the inflammatory processes that occur in IBD, the cytokine- and cell-mediated regulation of immunoglobulin production appears more important because of its ability to trigger the complement pathway and macrophage activation. A variety of processes may account for the large influx of macrophages and PMNs, including cytokines, such as generation of interleukin (IL)-1, IL-6, IL-8, IL-10, and tumor necrosis factor-α (TNFα), complement activation, and eicosanoid synthesis. Once the influx of macrophages and PMNs occurs, a markedly increased production of free oxygen radicals, proteases, LTs (in particular LTB_4), platelet-activating factor (PAF-acether), and other soluble mediators of inflammation results in the secondary amplification of the inflammatory response, which provides the clinical manifestations of the final inflammatory processes in the intestine (6). Although eicosanoids are far down the pathogenetic pathway of IBD and are relatively nonspecific, they are important because they may be considered key mediators of inflammation and belong to the portion that actually causes the damage to the intestine, in addition to being more readily approachable by drug therapy (6). This impression is emphasized by the fact that most of our current drug therapy, such as corticosteroids, anti-inflammatory salicylates, and immunosuppressive agents, inhibit the nonspecific inflammatory processes observed in IBD.

LEUKOTRIENE BIOLOGY

Eicosanoids are a family of molecules derived from arachidonic acid. The products of this pathway have been implicated in a large number of physiologic functions and disease processes (7). Among them are the cyclooxygenase products [prostaglandins (PGs), thromboxanes (TXs), and prostacyclin], the 5-LO products, including LTs, the products of 12- or 15-lipoxygenases, and lipoxins, which are products of 5- or 15-lipoxygenases. Both cyclooxygenase and lipoxygenases are enzymes that catalyze the stereospecific insertion of molecular oxygen into various positions in arachidonic acid. They are named according to the position of the carbon atom into which the oxygen is inserted. The insertion of oxygen provides the energy potential for this large family of molecules, and each of the reactions of arachidonic acid with oxygen results in a molecule with a unique spectrum

of biologic activities (8). Enzymes metabolizing arachidonic acid are selectively distributed in different cell types, thus further increasing the diversity of the eicosanoids that can be produced.

RATIONALE FOR INHIBITING LEUKOTRIENES IN IBD

Chronic IBD is associated with excess eicosanoid formation in the target tissue of inflammation (6). The first inflammatory mediator examined was PGE_2, which is produced in markedly increased amounts in IBD (9). However, the use of cyclooxygenase inhibitors appears to worsen IBD, to provoke a relapse (10), or even to induce ulcerative disease of the colon indistinguishable from ulcerative colitis (11). The explanation for this was not entirely clear until studies were carried out examining the inhibition of the 5-LO pathway by 5-aminosalicylic acid (5-ASA) and, more importantly, the markedly increased production of LTB_4 in IBD (12,13). It therefore appears very important which parts of the arachidonic acid metabolism pathway are blocked by pharmacologic agents.

It is now well established that LTs, and in particular LTB_4, are generated and released in vitro from colonic mucosa obtained from patients with ulcerative colitis or Crohn's disease (14,15). The large bowel also produces LTs in vivo, as assessed by rectal equilibrium dialysis in patients with active ulcerative colitis (16). Because these products are not produced by peripheral blood leukocytes in vitro after the addition of an activating stimulus, the responsible cells must be activated in situ. The rationale for using anti-LT drugs in IBD is based, therefore, on markedly increased generation of LTB_4, potent proinflammatory actions of LTB_4, and clinical efficacy of established drugs [corticosteroids, sulfasalazine, and its metabolite 5-aminosalicylic acid (5-ASA)], all of which, among other pharmacologic properties unrelated to LT biosynthesis, inhibit LT formation.

ANTILEUKOTRIENE AGENTS IN IBD

The compounds that inhibit LT formation and function can be conveniently separated into agents that reduce substrate arachidonic acid availability, agents that inhibit the 5-LO, and agents that blockade LT receptors (17,18).

Agents That Reduce Substrate Arachidonic Acid Availability

The basic strategies to block LT biosynthesis are shown in Fig. 1. The first enzymatic step in the biosynthesis of LTs in the inflamed mucosa is the liberation of free arachidonic acid from membrane phospholipids by the

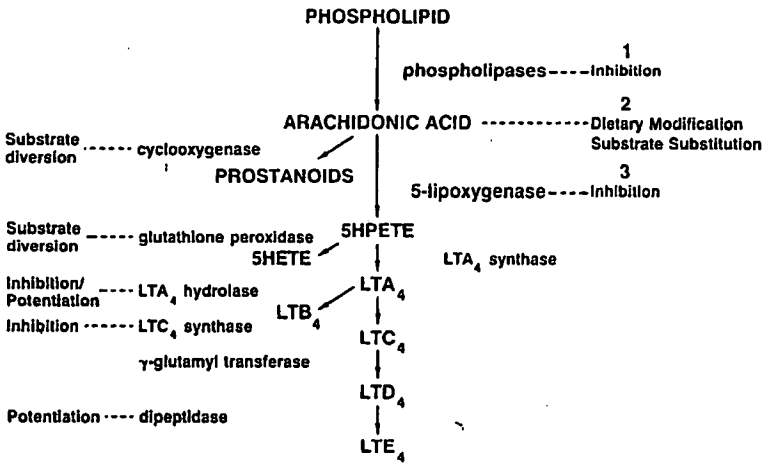

FIG. 1. The three enzymatic steps (right 1–3) that provide the basic strategies to block leukotriene (LT) biosynthesis.

hydrolytic enzyme phospholipase A_2 (18). Drugs that act at this point can generally be divided into three types: those interacting with phospholipase A_2; those interacting with the substrate for phospholipase A_2; and those modulating intracellular calcium levels. Most important are the corticosteroids, which belong to the first type, but they have no direct interaction with the enzyme. Instead, a receptor–drug complex is formed that induces synthesis and release of proteins with antiphospholipase properties (18). Thus, the corticosteroids prevent formation of free arachidonic acid by promotion of the phospholipase A_2 inhibitor lipocortin (19–23), and in patients with active IBD prednisolone causes a prompt reduction in the generation of LTB_4 and PGE_2 (13). However, corticosteroids also have effects on lymphocyte differentiation, cytokine synthesis, and interferon production (1,24, 25). It is therefore still unclear which of the pharmacologic properties account for the therapeutic efficacy of corticosteroids.

The second type includes a wide range of drugs, most of which have other actions and therefore inhibit phospholipase A_2 indirectly, such as the antimalarial agents mepacrine and chloroquine and the local anaesthetic lidocaine.

The third group comprises calcium antagonists, but the concentrations required far exceed those necessary for inhibition of "slow" calcium channels. Nevertheless, the calcium-channel antagonist verapamil has recently been shown to inhibit mucosal 5-LO activity in therapeutic concentrations by 30% in rectal biopsies from patients with active ulcerative colitis without

affecting mucosal PGE_2 release (26). This effect is explained by the calcium dependence of 5-LO. The observation awaits therapeutic evaluation.

Incorporation of alternative fatty acid substrates may also affect biosynthesis of LTs. If the dietary intake of arachidonic acid is decreased and/or the intake of eicosatrienoic and eicosapentaenoic acid is increased, LTs of the 3 and 5 series are produced instead. LTB_3 is only slightly less potent than LTB_4 as a chemotactic and chemokinetic agent of human PMNs, but the structurally analogous product LTB_5 (5,12-dihydroxyeicosapentaenoic acid) is less efficiently produced and is a markedly weaker stimulator of PMN function. As a consequence, the therapeutic potential of dietary precursor modulation by a fish oil-supplemented diet [ω-3 fatty acids, such as eicosapentaenoic acid (C20:5,ω-3) and docosahexaenoic acid (C22:6,ω-3)] in the treatment of IBD has been shown to result in a 35% to 50% decrease in PMN production of LTB_4 (27–29). Significant improvement in symptoms and histologic appearance of the rectal mucosa has been observed in several small series of patients with Crohn's disease (28) and ulcerative colitis (30–32) given fish oil (Max EPA) 3 to 4 g daily for 2 to 6 months in uncontrolled studies, as well as in small double-blind crossover studies in ulcerative colitis (33,34). However, a larger, randomized, double-blind trial comprising 96 patients with ulcerative colitis failed to reveal any benefit in remission maintenance or treatment of relapse on 4.5 g of eicosapentaenoic acid daily, although LTB_4 synthesis by blood peripheral PMNs in response to ionophore stimulation was reduced by 49%, 53%, and 59% after 2, 6, and 12 months, respectively (35). It should be emphasized, however, that the anti-inflammatory actions of fish oils, in addition to their inhibition of LTB_4, include suppression of IL-1 and PAF-acether synthesis and scavenging of free oxygen radicals. Several explanations for the lack of efficacy in remission maintenance exist. Probably more than 50% inhibition of LTB_4 biosynthesis is required to obtain clinical efficacy, but it is also possible that placebo olive oil has therapeutic activity or acts as a scavenger of free oxygen radicals (35). The results of an as yet unpublished study demonstrating that, e.g., concentrations of docosahexaenoic acid are abnormally high in patients with IBD may be more important, because these patients have an increased metabolism of polyunsaturated fatty acids and normal plasma levels of arachidonic acid and linoleic acid (personal communication). These observations, therefore, question the rationale for using fish oil in the treatment in IBD.

Agents That Inhibit 5-Lipoxygenase

At present, the greatest amount of effort is being assigned to the identification of compounds that specifically and effectively inhibit the 5-LO enzyme. In addition to calcium-channel antagonists mentioned above, sul-

TABLE 1. *Examples of experimental 5-lipoxygenase (5-LO) inhibitors*

Agent	Chemical structure	Selected references
Zileuton	*N*-(1-(benzo-(b)-thien-2-yl)ethyl)-*N*-hydroxyurea	76,77
BW 4AC	*N*-(3-phenoxycinnamyl)acetohydroxamic acid	35,94
BW A137C	*N*-(4-benzyloxybenzyl)acetohydroxamic acid	94
BW A797C	*N*-(3-(5,6,7,8-tetrahydro-2-naphthyl)prop-2-enyl)acetohydroxyamic acid	94
EP10045	Methyl-2-butyl-mercapto-4-catechol	95
L-651,392	4-Bromo-2,7-dimethoxy-3H-phenothiazin-3-one	96,97
MK-0591	3-(1-((4-chlorophenyl)methyl)-3((1,1-dimethyl-ethyl)thio)-5(quinolin-2-ylmethyloxy)-1H-indol-2-yl)-2,2-dimethyl-propanoate	
ONO-RS-085	*N*-(2E,11Z,14Z-eicosatrienoyl)-anthranilic acid	98
S-26431	3-(3,5-di-t-butyl-4-hydroxyanilino)-benzoic acid	99
SC41930	7-(3-(4-acetyl-3-methoxy-2-propylphenoxy)-propoxyl)-3,4-dihydro-8-propyl-2H-1-benzopyran-2-carboxylic acid	75,100

fasalazine and its active moiety 5-ASA (mesalazine) are also weak 5-LO inhibitors (15,24,36–42), but they may also reduce inflammation by modulating PMN leukocyte function (43–47) and by acting as inhibitors of PGs (9,48–50), TXs (51), PAF-acether (52), IL-1 (25,53), TNFα (54), intestinal mast cell and basophil-stimulated histamine release (55), and fMLP-receptor binding (56), in addition to being scavengers of free oxygen radicals (57–69). As was the case with corticosteroids, it is still unclear which of the above properties account for the therapeutic efficacy of sulfasalazine and 5-ASA.

The compounds identified as inhibitors of 5-LO can be divided into three main types. First, there are antioxidants (70). Classical examples comprise the phenyl pyrazoline BW755C, nordihydroguaiaretic acid, and vitamin E (7). They do inhibit the 5-LO enzyme, but it should be recognized that they are not well-defined, selective enzyme inhibitors but rather are dual inhibitors of the cyclooxygenase and the 5-LO enzyme. Second, there are substrate analogues, such as 5,6-dehydro-arachidonic acid. Third, there is a large miscellaneous group of inhibitors with differing mechanisms of action (Table 1), among which hydroxamic acids (71,72) are potent and more selective inhibitors of 5-LO, but 20 to 30 times less potent as cyclooxygenase inhibitors.

Experimental 5-Lipoxygenase Inhibitors

A number of 5-LO inhibitors have been studied in animal models. Although no model of IBD exactly mimics human disease, many features of the models can be used to investigate the role of particular inflammatory cells and mediators in the underlying inflammatory processes. However, studies with 5-LO inhibitors in models of IBD have yielded variable effects, but this notion also applies to the effects of corticosteroids and sulfasalazine (73). Hence, the relevance of models for selection of novel therapeutic agents should be viewed with caution, although such studies may allow selection of potent, selective, and bioavailable 5-LO inhibitors for clinical evaluation.

In vitro data on human colitis tissue have also begun to accumulate. The acetohydroxamic acid BWA4C inhibited basal and ionophore stimulated in vitro formation of LTB_4 synthesis by colorectal biopsy specimens obtained from patients with ulcerative colitis, without significantly inhibiting PGE_2 or TXB_2 synthesis (74). The IC_{50} for inhibition of LTB_4 formation by BWA4C was 0.03 μmol/L compared with an IC_{50} of 0.08 μmol/L for nordihydroguaiaretic acid. In a fairly similar design, SC41930 and SC45662 inhibited mucosal LTB_4 release by 17% and 41%, respectively, compared with an inhibition by 5-ASA of 18% (75).

Until now, clinical data on 5-LO inhibition in patients with IBD are available only for zileuton (Abbott-64077). Another compound, MK-886, was withdrawn from clinical development pending evaluation of observed animal toxicity but has already been replaced by MK-0591, which is presently undergoing phase I clinical evaluation in patients with mild-to-moderate ulcerative colitis (personal communication). MK-0591 is a potent LTB_4 biosynthesis inhibitor that binds to the 5-LO-activating protein (FLAP) and thereby inhibits the translocation of 5-LO. The compound appears able to inhibit calcium ionophore-stimulated LTB_4 biosynthesis ex vivo in whole blood by more than 99% after a single oral dose of 125 mg MK-0591 in healthy volunteers, and 24 hr after dosing the mean percentage inhibition of a single 500-mg dose was still more than 90% (personal communication).

Zileuton

Zileuton (Table 1) strongly inhibits 5-LO activity in rat basophilic leukemia-1 cells and in rat and human PMNs. In rat leukocytes the drug is 28-fold more potent in inhibiting 5-LO than cyclooxygenase (76).

In a single-dose study, LTB_4 and PGE_2 concentrations in rectal dialysis fluid from 10 patients with active ulcerative colitis were measured before and after oral administration of 800 mg of zileuton (76). Figure 2 shows that the median LTB_4 level fell significantly, from 4.9 (range 0.6 to 20.4) ng/ml before treatment to 1.6 (0.3 to 5.7) ng/ml after 4 hr and 0.7 (0.1 to 8.0) ng/ml after 8 hr; it returned to pretreament levels after 24 hr. The concentrations of PGE_2 did not change significantly. These findings in patients with ulcerative colitis indicate that zileuton is a specific (i.e., without effect on the cyclooxygenase) and efficient inhibitor of LTB_4 formation in the target tissue of inflammation (76).

Two studies on the use of zileuton in the treatment of ulcerative colitis have been published. First, in an open trial patients given zileuton 800 mg once a day for 4 weeks showed a trend toward symptomatic improvement (77). Second, the results of a randomized, double-blind, placebo-controlled, multicenter trial [the first of two scheduled interim analyses (78)] of zileuton 800 mg orally twice daily for up to 4 weeks in patients with mild-to-moderate

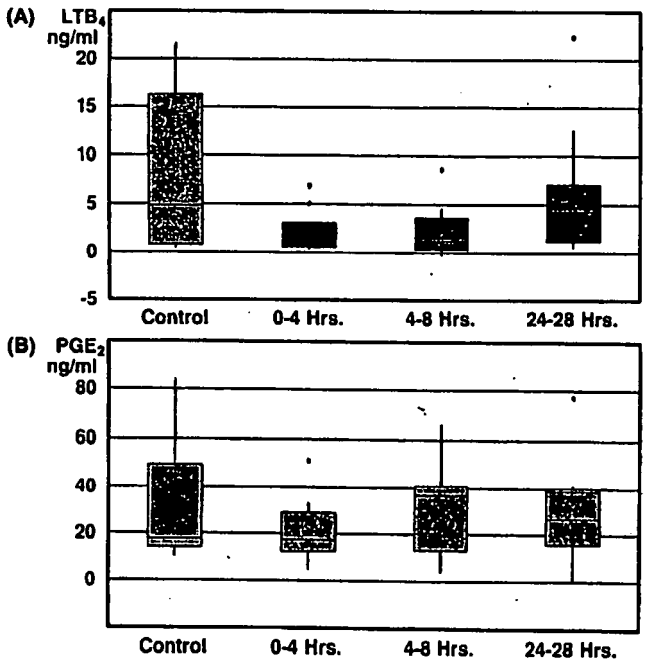

FIG. 2. Box plots of LTB_4 (A) and PGE_2 (B) concentrations in rectal dialysates from 10 patients with mild to moderately active ulcerative colitis before (control) and after administration of a single dose (800 mg) of zileuton. The central horizontal line in each box represents the group median, the box encloses the middle 50% of the data values, and the tails extend to the most extreme values within a border or "fence" at 1.5 times the interquartile range. The dots denote outliers. Adapted from ref. 76.

disease activity showed that zileuton treatment resulted in significant improvement in symptoms, in addition to significant inhibition of LTB_4 in the target tissue of inflammation (79). Briefly, a primary stratification was performed for patients with (n = 45) or without (n = 31) concomitant sulfasalazine or 5-ASA. The patients were evaluated before entry, after 2 and 4 weeks, and at follow-up after week 5. Efficacy evaluation was based on symptoms, sigmoidoscopy, and histology. The safety assessments included physical examination and laboratory screening. Seventy-six patients entered the study. Forty patients were randomized to zileuton and 36 to placebo. A clinical response with combined improvement in both symptom and sigmoidoscopy scores occurred more often in the zileuton group and significantly so in the subset of patients without concurrent sulfasalazine. Furthermore, zileuton significantly improved total symptom score and scores of rectal

bleeding, stool character, abdominal/rectal pain, and urgency. A statistically significant improvement in histology score was also noted when patients receiving zileuton without concomitant sulfasalazine were compared with those receiving placebo. No adverse events or toxicity indicating an unfavorable benefit-to-risk ratio were observed. The LTB_4 concentrations in rectal dialysates were significantly inhibited by zileuton, with a median percentage inhibition of 70%.

These data offer encouraging therapeutic insights into the control of the inflammatory response in IBD, but a higher degree of 5-LO inhibition and a prolonged treatment should be assessed in future studies. Nevertheless, the results provide direct evidence for the hypothesis that selective inhibition of the synthesis of a single mediator may result in clinical response, although more than a hundred different inflammatory mediators are involved in the pathogenesis of ulcerative colitis (6).

Agents That Blockade Leukotriene Receptors

Antagonism of the actions of LT can also be obtained by using selective receptor antagonists (80,81). The number and specificity of LT receptors remain a matter of debate (82–84). Since the discovery of the first LT antagonist, FPL 55712 (85), several new agents have been described. Most experience has been obtained with cysteinyl LT-receptor antagonists, the LTD_4 receptors being considered an important target for pharmacologic intervention. Less effort has been given to the search for LTB_4 antagonists because of the complexity of the in vitro assays (86), among other things owing to changes in receptor expression modulated by protein kinase C (87). Such changes may account for changes in PMN responsiveness to LTB_4.

Some agents with purported LT receptor antagonism [e.g., CGP 34064A (88) and CGP 35949 (89)] not only block the action of LTs at the receptor level but also block the biosynthesis of the LTs by inhibiting phospholipase A_2. Furthermore, some of the antagonists also possess 5-LO inhibitory activity [e.g., FPL 55712 (90), SC41930 (75), and Wy-49911 (90)].

CONCLUSIONS AND PROSPECTS

A large number of anti-LT drugs are presently under development. Selective interventions that target other mediators of inflammation [e.g., PAF-acether receptor antagonists (91–93), IL-1–receptor antagonists, in addition to antibodies to IL-1 or IL-$1R_S$, and bradykinin-receptor antagonists (93)] will soon follow. In this connection it should be emphasized that blocking 5-LO is a highly effective way of inhibiting cytokine synthesis. In addition, several kinin antagonists were synthesized years ago, and it was shown that B_2-receptor antagonists (D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-D-Phe-Thi-Arg-

TFA and D-Pro-Phe-Arg-heptylamide) cause significant antagonism of bradykinin-induced inflammatory reactions (93). However, medical intervention focusing on inhibition of the 5-LO, LT receptors, the terminal enzymes responsible for the formation of LTB_4, cysteinyl LTs, and PAF-acether receptors presently appears the most promising therapeutic approach in IBD. The main disadvantage of existing new LT inhibitors relates to the high potency of the LTs. Unless a high degree of inhibition can be achieved, endogenous LTs may still be present in sufficient amounts to produce their effects. Because a physiologic role for LTs is generally accepted, the possibility that effective long-term inhibition may be associated with side effects should also be ruled out before placing regulation of the LT pathway within the realm of clinical medicine.

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Summary: Eicosanoid Effector Sites

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The previous five chapters, with their diverse topics, cannot be easily integrated under a unitary theme. However, one area of unanswered questions is: Do 5-lipoxygenase products or other oxygenated arachidonic acid metabolites have intracellular regulatory functions in addition to their effector functions as intercellular mediators?

In the process of assessing the distal products of the 5-lipoxygenase pathway, i.e., the cysteinyl leukotrienes and LTB_4, the potential biological actions of the less stable precursors, 5-HPETE and LTA_4, or of other oxygenated arachidonic acid metabolites, such as EETs (1), are often forgotten. It has been shown, for example, by Chang et al. (2) that tumor necrosis factor-α-dependent induction of manganous superoxide dismutase in a murine adipogenic cell line TA_1 is dependent on 5-HPETE production. The mechanism by which this effector function occurs appears to require the induction of *c-fos* transcription by the lipid hydroperoxide; the induction does not appear to be regiospecific and thus apparently reflects the effector function of the hydroperoxy domain rather than a more complicated signal specifically related to the fact that it is a 5- rather than a 12- or 15-lipoxygenase product. Likewise, Peppelenbosch and colleagues (3) have shown that epidermal growth factor brings about a 5-lipoxygenase-dependent transcription of the early response gene *junB* in P19 8-39 cells (murine P19 embryonal carcinoma cells stably transfected with human EGF receptor).

Similarly, the role of noncyclooxygenase products of arachidonic acid as modulators of the growth response to mitogenic agents may rely on their intracellular functions as stimulatory factors for gene activation. This can be concluded from a study by Sellmayer et al. (4) in which increased mRNA levels of the immediate early response genes *c-fos* and Egr-1 in proliferating mesangial cells have been reported in association with increased levels of i.c. EET-like compounds. Results by Bernstrom et al. (5) imply that such

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Exhibit 6

Omega-3 fatty acids suppress the enhanced production of 5-lipoxygenase products from polymorph neutrophil granulocytes in cystic fibrosis

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Abstract. Pulmonary damage in cystic br osis (CF) is associated with chronic inflammation mediated in part by proinflammatory 5-lipoxygenase products (5-LOP, leukotrienes and 5-hydroxyeicosatetraenoic acid) from polymorph neutrophil granulocytes (PMN). The authors studied 5-LOP formation of PMN from CF patients and *in vitro* effects of added eicosapentaenoic acid (EPA) and fish oil. Circulating PMN were isolated from 10 CF patients without acute infections and 10 control persons of the same age (4–20 years). Total 5-LOP liberation from PMN of CF patients was significantly increased over controls after incubation with the calcium ionophore A23 (1 μmol L^{-1}) without arachidonic acid (AA) (380 ± 24 vs. 294 ± 28 pmol mL^{-1}) and with 10 μmol L^{-1} AA (1303 ± 104 vs. 1015 ± 104 pmol mL^{-1}), and there were non-significant trends to high values after incubation with 5 μmol L^{-1} platelet activating factor (PAF, 134% of controls) and 1 μmol L^{-1} formyl-methionyl-leucyl-phenylalanine (FMLP, 125%). The addition of 100 μg mL^{-1} fish oil to PMN of CF patients challenged with A23 completely suppressed synthesis of proinflammatory 5-LOP of the 4-series, while inactive 5-LOP metabolites of the 5-series were produced. Added EPA (10 μmol L^{-1}) also suppressed 4-series 5-LOP and significantly reduced leukotriene B_4 concentration by 48% from 39·9 ± 3·2 to 20·6 ± 11·4 pmol L^{-1}, again with a concomittant increase of inactive 5-series metabolites. The authors conclude that the turnover of endogenous and exogenous AA is enhanced in CF, possibly due to stimulated phospholipase A_2 activity. The relatively small effect of the receptor dependent stimuli PAF and FMLP may be caused by a down-regulation of PMN receptors in CF. Supplementation of long-chain ω-3-fatty acids may be beneficial for reducing excessive inflammation in CF patients and should be further evaluated.

Keywords. Cystic fibrosis, eicosapentaenoic acid, fish oil, 5-lipoxygenase products, polymorph neutrophil granulocytes.

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Introduction

The median age of survival and the quality of life of patients with cystic fibrosis (CF) has improved considerably over the last few decades, but the patients' prognosis remains limited by the progressive deterioration of lung structure and function that is associated with chronic pulmonary inflammation. These processes are triggered by proinflammatory mediators, including complement factors, immune complexes, oxygen radicals and the products of arachidonic acid (20:4ω-6, AA) formed by the enzyme 5-lipoxygenase [1,2]. The proinflammatory 5-lipoxygenase products (5-LOP) leukotriene B_4 (LTB_4) and 5-hydroxy-eicosatetraenoic acid (5-HETE) are potent activators of granulocyte functions such as chemotaxis and degranulation [3,4]. We hypothesized that 5-LOP promote pulmonary inflammation in CF. Therefore, we investigated the production of 5-LOP after *in vitro* challenge of polymorph neutrophil granulocytes (PMN) from CF patients and a control group. Eicosapentaenoic acid (20:5ω-3, EPA) contained in salt water fish is a strong metabolic competitor of arachidonic acid [5,6], and adjuvant treatment with fish oil preparations has been used to suppress activity of inflammatory and autoimmune disorders, including arthritis, psoriasis and ulcerative colitis [4,6]. Therefore, we studied whether 5-LOP formation of PMN from CF patients can be suppressed by eicosapentaenoic acid and by a fish oil preparation containing EPA.

Patients and methods

Ten CF patients treated as outpatients at the Kinderpoliklinik, University of Munich aged between 4 and 20 years (13·4 ± 3·9 years, M ± SD; seven male) volunteered to participate in this study. The diagnosis of CF was confirmed by two positive sweat tests in all patients. All participating patients were in a stable general condition, free of acute infection and breathed spontaneously without the need of supplemental oxygen. Nine patients had sputum cultures positive for *Pseudomonas* infection. Lung function analysis indicated a vital capacity of 84·1 ± 14·2% (M ± SD)

of values expected for weight and length, and a forced expiratory volume (FEV_1) of 77·3 ± 23·7%. No patient took any corticoids or other anti-inflammatory drugs for at least 8 weeks prior to the examination. The control group consisted of 10 generally healthy children and adolescents aged between 4 and 20 years (10·8 ± 3·3 years; five male) without acute infection treated for elective oropharyngeal surgery at this hospital, in whom blood samples were obtained when venipunctures were performed for diagnostic reasons prior to surgery. All subjects in the CF and in the control groups had a C reactive protein (CRP) $<2\,mg\,dL^{-1}$ and a leucocyte count $<10000\,\mu L^{-1}$ at the time of study. The study protocol was approved by the ethics committee of the medical faculty, University of Munich, and informed consent was obtained from the probands and their parents. The volume of the venous blood sample obtained in each subject was 9 mL.

The standard substances 20-OH–LTB_4, 20-COOH–LTB_4, LTB_4, 6-*trans*-LTB_4, 6-*trans-epi*-LTB_4, 5,6-diHETE, 5,6-*epi-di*-HETE and 5-HETE were purchased from Paesel AG (Frankfurt/Main, Germany). The stimuli A23187 (A23), PAF, FMLP as well as arachidonic acid, eicosapentaenoic acid and lipoprotein lipase from *Pseudomonas* were obtained from Merck (Darmstadt, Germany). A 10% fish oil emulsion developed for parenteral infusion was kindly provided by Fresenius AG (Oberursel, Germany). The chromatographic agents as well as RPMI (Roswell Park Memorial Institute) 1640 medium with 10% fetal calf serum were purchased from Fluka KG (Heidelberg, Germany), an octadecylsilyl column with 5 μm particle size from Gynkothek (Germering, Germany), C18 Sep Pack Bond Elut columns from Varian (Harbor City, USA) and Percoll from Pharmacia Fine Chemicals (Uppsala, Sweden).

Polymorph neutrophil granulocytes (PMN) were isolated from 9 mL venous heparinized blood with a discontinuous gradient centrifugation [7]. The purity of the granulocyte fraction was >86·9% by microscopy. After 60–90 min incubation in RPMI 1640 medium with 10% fetal calf serum and two following washing procedures with Hanks/Hepes buffer, 1 ml each of cell suspensions containing $2·5 \times 10^6$ cells from 10 CF patients and 10 controls were incubated at 37°C for 10 min with A23 ($1\,\mu mol\,L^{-1}$) with and without added arachidonic acid (AA, $10\,\mu mol\,L^{-1}$), with PAF ($5\,\mu mol\,L^{-1}$) and AA ($10\,\mu mol\,L^{-1}$), and with FMLP ($1\,\mu mol\,L^{-1}$) and AA ($10\,\mu mol\,L^{-1}$). The PMN of five CF patients were also stimulated with A23 ($1\,\mu mol\,L^{-1}$) with added eicosapentaenoic acid (EPA, $10\,\mu mol\,L^{-1}$) or added fish oil emulsion ($100\,\mu g$ triglycerides mL^{-1} equal to 5 mmol EPA L^{-1}) with lipoprotein lipase from *Pseudomonas* ($0·3\,mg\,mL^{-1}$). The liberated 5-lipoxygenase products (5-LOP) were extracted with an octadecyl column (Hypersil with 5 μm particle size) with methanol/water/acetic acid (72/28/0·16 by volume, pH 4·9) and separated by isocratic reversed phase HPLC as described previously [8]. A Rheodyne syringe loading sample injector (model 7125), a Gynkothek 600 pump, a Spectroflow 773 UV-detector and a Shimadzu Gynkothek integrator CR6A were used for HPLC. The metabolites OH/COOH–LTB_4, 6-*trans*/6-*trans-epi*-LTB_4, LTB_4, 5,6-/5,6-*epi-di*-HETE were detected at a wavelength of 270 nm and 5-HETE at 237 nm. The recovery of added standards was 60–82%. The results of the CF and control groups were compared by an unpaired two-sided *t*-test with the program Minitab extended Vers. 8 for the PC [9]. The level of statistical significance was set at $P < 0·05$.

Results

The stimulation of PMN with the calcium ionophore A23 resulted in a significantly higher synthesis of total 5-LOP in CF patients than in controls (Table 1). There was a significant increase in the non-enzymatic products OH/COOH–LTB_4, 6-*trans*/6-*trans-epi*-LTB_4 and 5,6/5,6-*epi-di*-HETE, whereas the enzymatically produced metabolites LTB_4 and 5-HETE did not

Table 1. Concentrations of 5-lipoxygenase products of the 4-series liberated from polymorph neutrophil granulocytes of 10 cystic fibrosis (CF) patients and 10 controls (pmol mL^{-1}, M ± SE; $^*P < 0·05$) after *in vitro* challenge with the calcium ionophore A23 ($1\,\mu mol\,L^{-1}$) or with A23 and added arachidonic acid ($10\,\mu mol\,L^{-1}$), and the difference between A23 challenges with and without added arachidonic acid

	Challenge with A23		Challenge with A23 + AA		Difference (A23 + AA)-A23	
	CF patients	Controls	CF patients	Controls	CF patients	Controls
OH/COOH-LTB_4	95·4 ± 10·0	63·1 ± 7·6*	147·3 ± 26·0	85·7 ± 8·0*	51·9 ± 0·6	22·61 ± 0·47*
6-*tr*-LTB_4	48·3 ± 3·5	35·8 ± 3·2*	167·0 ± 18·0	99·8 ± 9·4*	118·7 ± 0·9	64·00 ± 0·45*
6-*tr-epi*-LTB_4	49·3 ± 3·5	37·1 ± 3·7*	172·1 ± 19·0	105·7 ± 10·0*	122·8 ± 1·3	71·60 ± 0·45*
LTB_4	55·9 ± 4·7	49·5 ± 4·9	78·3 ± 11·0	60·0 ± 5·5	22·4 ± 0·4	10·50 ± 0·05
5,6-di-HETE	12·8 ± 1·1	9·4 ± 0·8*	26·1 ± 3·6	14·6 ± 1·5*	13·0 ± 0·2	5·2 ± 0·1*
5,6-*epi*-di-HETE	7·7 ± 0·8	6·2 ± 0·6	20·1 ± 3·1	10·8 ± 1·2*	12·5 ± 0·3	4·5 ± 0·1*
5-HETE	110·3 ± 11·0	96·8 ± 12·0	692·0 ± 34·0	635·0 ± 86·0	582·0 ± 7·80	538·2 ± 6·8
total 5-LOP	379·7 ± 24·0	294·0 ± 28·0*	1303·0 ± 104·0	1015·0 ± 104·0*	915·00 ± 7·00	721·0 ± 7·2*

LTB_4 = leukotriene B_4; OH/COOH-LTB_4 = hydroxyl/carboxyl-LTB_4 (omega-oxidation products); 6-*tr*-LTB_4 = 6-*trans*-LTB_4; 6-*tr-epi*-LTB_4 = 6-*trans-epi*-LTB_4; 5-HETE = 5-hydroxyeicosatetraenoic acid; 5,6-diHETE = 5,6-di-HETE; 5,6-*epi*-diHETE = 5,6-*epi-di*-HETE; 5-LOP = 5-lipoxygenase products.

Table 2. Concentrations of 5-lipoxygenase products of the 4-series liberated from polymorph neutrophil granulocytes of 10 cystic fibrosis (CF) patients and 10 controls (pmol mL^{-1}, M ± SE, $^*P < 0.05$) after *in vitro* challenge with the ligand operating stimuli platelet activating factor (PAF, 5 μmol L^{-1}) and formyl-methionyl-leucyl-phenylalanine (FMLP, 1 μmol L^{-1}) with added arachidonic acid (AA, 10 μmol L^{-1})

	Challenge with PAF + AA		Challenge with FMLP + AA	
	CF patients	Controls	CF patients	Controls
OH/COOH-LTB_4	42·9 ± 5·3	33·4 ± 4·2	37·2 ± 4·0	28·5 ± 4·7
6-*tr*-LTB_4	88·5 ± 8·9	63·0 ± 2·0	10·2 ± 1·5	6·7 ± 1·1
6-*tr*-*epi*-LTB_4	91·5 ± 9·5	64·7 ± 12·0	9·79 ± 1·5	6·1 ± 1·0
LTB_4	88·0 ± 5·6	76·1 ± 8·4	33·1 ± 3·5	22·8 ± 4·4
5,6-di-HETE	13·9 ± 1·7	9·7 ± 2·0	3·7 ± 0·5	2·6 ± 0·5
5,6-*epi*-di-HETE	12·6 ± 1·1	8·4 ± 0·5*	4·0 ± 0·6	2·6 ± 0·4
5-HETE	577·0 ± 9·0	425·0 ± 7·4	136·0 ± 15·0	117·3 ± 20·0
total 5-LOP	915·0 ± 59·0	681·0 ± 109·0	233·3 ± 20·0	186·7 ± 32·0

LTB_4 = leukotriene B_4; OH/COOH-LTB_4 = hydroxyl/carboxyl-LTB_4 (omega-oxidation products); 6-*tr*-LTB_4 = 6-*trans*-LTB_4; 6-*tr*-*epi*-LTB_4 = 6-*trans*-*epi*-LTB_4; 5-HETE = 5-hydroxyeicosatetraenoic acid; 5,6-diHETE = 5,6-di-HETE; 5,6-*epi*-diHETE = 5,6-*epi*-*di*-HETE; 5-LOP = 5-lipoxygenase products.

differ significantly from controls. The challenge of PMN with A23 and added AA resulted in a significantly larger increase of the non-enzymatically produced 5-LOP (OH/COOH-LTB_4, 6-*trans*/6-*trans*-*epi*-LTB_4 and 5,6/5,6-*epi*-*di*HETE) as well as total 5-LOP in the CF-group than in controls (Table 1). The increase of 5-LOP liberation induced by added AA, expressed as the difference between A23 challenges with and without AA, was also markedly and significantly higher in PMN from CF patients than controls (Table 1). In contrast, stimulation with the receptor dependent stimuli PAF and FMLP with added AA led only to an increasing trend of 5-LOP production, with a significant difference only for PAF induced liberation of 5,6-*epi*-*di*-HETE (Table 2).

PMN of five CF patients could be challenged with A23 and added fish oil (100 μg) and lipoprotein lipase, which led to a complete suppression of the synthesis of proinflammatory 5-LOP of the 4-series, whereas the addition of free EPA (10 μmol) reduced 5-LOP liberation by about one third, as compared to stimulation with A23 alone (Table 3). Instead, 5-LOP metabolites of the 5-series with little inflammatory activity, which were not found at stimulation with A23 alone, were produced at remarkable concentrations when fish oil or EPA were added (Table 4).

Discussion

This study demonstrates an enhanced formation of 5-LOP in isolated PMN from CF patients. 5-LOP of the 4-series are potent proinflammatory lipid mediators [2,4,10,11], which may further the chronic pulmonary inflammation and destruction typical for CF patients. In contrast to the calcium ionophore A23, the receptor dependent stimuli PAF and FMLP had only a smaller effect on 5-LOP formation, which may be explained by a down-regulation of PMN-receptors in CF, possibly resulting from chronic inflammatory processes. The observed increased 5-LOP production

Table 3. Concentrations of 5-lipoxygenase products of the 4-series liberated from polymorph neutrophil granulocytes of five cystic fibrosis (CF) patients (pmol mL^{-1}, M ± SE, n.d. = not detectable; $^*P < 0.05$ vs. A23 alone) after *in vitro* challenge with A23 alone, with addition of 100 μg of a fish oil emulsion providing 5 mmol eicosapentaenoic acid (EPA) plus 0·3 mg lipoprotein lipase, or with addition of 10 μmol free EPA. Addition of fish oil completely suppressed proinflammatory 5-LOP, while EPA reduces the concentrations by about one-third

Challenge with:	A23 alone	A23 + 100 μg fish oil	A23 + 10 μmol EPA
OH/COOH-LTB_4	62·6 ± 7·5	n.d.	40·2 ± 21·0
6-*tr*-LTB_4	34·5 ± 2·3	n.d.	30·4 ± 2·7
6-*tr*-*epi*-LTB_4	35·8 ± 2·3	n.d.	25·4 ± 0·9
LTB_4	39·9 ± 3·2	n.d.	20·6 ± 11·4*
5,6-di-HETE	9·9 ± 0·9	n.d.	ND
5,6-epi-di-HETE	8·9 ± 0·6	n.d.	ND
5-HETE	84·2 ± 19·9	n.d.	70·1 ± 7·3
Total 5-LOP	275·9 ± 11·2	n.d.	281·0 ± 12·0

LTB_4 = leukotriene B_4; OH/COOH-LTB_4 = hydroxyl/carboxyl-LTB_4 (omega-oxidation products); 6-*tr*-LTB_4 = 6-*trans*-LTB_4; 6-*tr*-*epi*-LTB_4 = 6-*trans*-*epi*-LTB_4; 5-HETE = 5-hydroxyeicosatetraenoic acid; 5,6-diHETE = 5,6-di-HETE; 5,6-*epi*-diHETE = 5,6-*epi*-*di*-HETE; 5-LOP = 5-lipoxygenase products.

Table 4. Concentrations of practically inactive 5-lipoxygenase products of the 5-series liberated from polymorph neutrophil granulocytes of five cystic fibrosis (CF) patients (pmol mL^{-1}, M ± SE) after *in vitro* challenge with A23 with addition of 100 μg fish oil providing 5 mmol eicosapentaenoic acid (EPA) plus 0·3 mg lipoprotein lipase, or with addition of 10 μmol EPA. Added fish oil and EPA led to enhanced production of inactive 5-series 5-LOP that is not found with A23 alone

Challenge with:	A23 + 100 μg fish oil	A23 + 10 μmol EPA
OH/COOH-LTB_5	124·3 ± 12·5	40·2 ± 21·0
6-*tr*-LTB_5	53·8 ± 12·2	96·7 ± 23·3
6-*tr*-*epi*-LTB_5	45·2 ± 10·5	75·6 ± 18·2
LTB_5	11·0 ± 2·3	17·4 ± 4·9
5,6-di-HEPE	10·5 ± 3·4	15·9 ± 8·7
5,6-epi-di-HEPE	15·1 ± 4·3	11·4 ± 3·6
5-HEPE	319·4 ± 51·5	271·9 ± 75·3
Total 5-LOP	579·3 ± 44·5	493·4 ± 66·3

LTB_5 = leukotriene B_5; OH/COOH-LTB_5 = hydroxyl/carboxyl-LTB_5 (omega-oxidation products); 6-*tr*-LTB_5 = 6-*trans*-LTB_5; 6-*tr*-*epi*-LTB_5 = 6-*trans*-*epi*-LTB_5; 5-HEPE = 5-hydroxyeicosapentaenoic acid; 5,6-diHEPE = 5,6-di-HEPE; 5,6-*epi*-diHEPE = 5,6-*epi*-di-HEPE; 5-LOP = 5-lipoxygenase products.

after stimulation with A23 can be explained by an enhanced turnover of endogenous arachidonic acid (AA), presumably related to an activation of phospholipase A_2 with consecutive liberation of AA from membrane lipids [12]. This hypothesis is supported by the observation that phospholipase A_2 is less suppressed by corticoids in lymphocytes of CF patients than in controls [13]. In addition, we assume the activity of the cytochrome P450 system to be enhanced because of the observed significant increase of the ω-oxidation products of LTB_4 (OH/COOH-LTB_4) formed by this pathway. Since addition of free exogenous AA to PMN challenged with A23 led to a marked enhancement of 5-LOP synthesis, we conclude that the turnover of exogenous AA is also increased. This enhanced AA turnover may be one contributing factor to explain that CF patients both with and without exocrine pancreatic insufficiency typically show a pronounced depression of AA levels in their plasma and tissue lipids, with the relative reduction usually being more marked than that of the precursor fatty acid linoleic acid [14–16].

The effective suppression of proinflammatory 4-series 5-LOP by the fish oil emulsion used here and by EPA, with concomittant production of practically inactive 5-series metabolites, indicates a preferential metabolism of the long-chain ω-3 fatty acid EPA by the 5-lipoxygenase pathway [17]. The resulting concentrations of metabolites produced may also be influenced by the relatively slow transformation of LTA_5 to LTB_5 by LTA_5-hydrolase, as compared to LTA_4 transformation [3]. The *in vitro* concentration of 100 μg fish oil ml^{-1} used in this experiment is of the same order of magnitude as ω-3 fatty acid concentrations in plasma lipids following oral administration of ω-3-enriched diets [18,19]. Since 5-LOP of the 5-series have practically no proinflammatory activity [10,11], it appears conceivable that an adjuvant supplementation of fish oil preparations or of long-chain ω-3 fatty acids may be beneficial for CF patients by reducing excessive inflammation. In a cross-sectional study in 19 CF patients, Gibson and coworkers [20] found a significant positive correlation of long-chain ω-3 fatty acid contents in plasma phospholipids to lung function, as assessed by the 1 s forced expiratory volume (FEV_1). Recent results of a short-term interventional trial in 16 CF patients supplementing fish oil or placebo over 6 weeks, providing a daily dosage of 2·7 g EPA with the active preparation, showed a significant reduction of chemotactic activity of polymorph neutrophil granulocytes, sputum production and disease activity expressed by the Shwachman score, while improvements were found for lung function parameters (FEV_1 and vital capacity) [21]. In view of the marked effects of added arachidonic acid on *in vitro* production of 5-LOP, it is conceivable that not only the absolute plasma and tissue concentrations of ω-3 fatty acids but also the relative ratios between AA and EPA are of major importance for biological effects. Longer term studies on ω-3 fatty acid supplementation in CF patients, with close observation of biochemical and clinical effects, are needed to evaluate its therapeutic potential.

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Exhibit 17

THE DETECTION OF 5-LIPOXYGENASE AND CYCLO-OXYGENASE PRODUCTS IN SPUTUM OF PATIENTS WITH CHRONIC BRONCHITIS AND BRONCHIECTASIS

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ABSTRACT

Leukotrienes (LTs) and prostanoids (Ps) were detected in sputum of patients with chronic bronchitis and/or bronchiectasis (CB/B) using selective superfusion bioassay and radioimmunoassay (RIA) techniques.

Analysis of sputum extracts showed a 4-fold increase in the level of LTB_4 compared to the cysteinyl-containing LTs (LTC_4/LTD_4).

The measurement of cyclo-oxygenase products (COPs) indicated relatively greater amounts of the vasodilator prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) compared to the vasoconstrictor prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane A_2 (TxA_2) agents (70:30% of total COPs respectively).

The presence of eicosanoids (LTs and Ps) in sputum of patients with CB/B suggest that these biologically active substances may act as mediators of bronchoconstriction and inflammation in these diseases.

INTRODUCTION

Chronic bronchitis and bronchiectasis are pulmonary diseases in which patients expectorate sputum and frequent infective episodes supervene.

In B there is abnormal irreversible dilation of at least some bronchi in the affected areas, mucus transport is defective and repeated local infections occur. CB is a generalised disorder in which there is increased mucus production from hypertrophied mucus producing cells and defective ciliary clearance of mucus leading to recurrent

infections; most cases of CB are associated with smoking.

There is an overlap between these two pulmonary disorders since many patients with CB have bronchiectatic areas and a considerable number of patients with B are smokers and have generalised airway inflammation.

The sputum contains abnormal mucus and many different cell types, including different classes of leukocytes and macrophages which are capable of synthesizing LTs and Ps from their common precursor arachidonic acid (AA) via 5-lipoxygenase and cyclo-oxygenase pathways respectively (1, 2). These oxygenated products of AA metabolism have a wide range of pharmacological actions (3) which may be relevant to the pathological processes associated with CB/B.

LTs have already been identified in sputum extracts obtained from individuals with various pulmonary disorders (4, 5) but the measurement of both 5-lipoxygenase and cyclo-oxygenase products has not previously been reported.

The purpose of this study was to detect LTs and Ps in CB/B sputum by specific superfusion bioassay or RIA techniques to help in the evaluation of their possible role as mediators in these diseases.

METHODS

Patients. Fifteen patients with either CB and/or B were studied. The group consisted of 11 males and 4 females with a mean age of 60.7 years (range: 42-80).

All the CB subjects had been, or currently were, heavy smokers and conformed to the WHO definition of CB (6). All had severe, largely irreversible, airflow obstruction, sputum samples were collected during episodes of acute infective exacerbations.

The B subjects were diagnosed on clinical and radiological criteria based on a history of production of large volumes of purulent sputum, persistent localised coarse crackles on auscultation of the chest and radiographic evidence of B consisting of bronchial wall thickening with ring and tramline shadows. It was not felt to be justifiable to submit patients to bronchography to confirm the clinical diagnosis of B.

Assessment of lung function by the forced expiratory volume in one second (FEV_1) indicated severely impaired lung function with all subjects having an FEV_1 of less

than 50% of predicted.

Extraction and Purification. During chest physiotherapy, sputum was collected directly into ethanol on ice. After adjusting the final alcohol concentration to 80%, each sample was homogenised on ice for 2 mins using an Ultra Turrax homogeniser (Model TP 18/10) and centrifuged (MSE: 12,000g for 20 min at 4°C) to remove insoluble material. The supernatant was decanted and evaporated to dryness under reduced pressure using a rotorevaporator R110. The dried residue was then resuspended in 1ml of distilled water and partially purified by C_{18}-Sep Pak cartridges and millipore filters (Waters 0.45µm) as previously described (5).

Samples were stored under nitrogen at -20°C and resuspended in 1ml of distilled water for subsequent analysis by superfusion bioassay and RIA.

Bioassay of leukotrienes. Leukotrienes were assayed biologically using a combination of guinea-pig lung parenchymal strip (GPP) and ileum smooth muscle (GPISM). The use of these two tissues allows discrimination between the cysteinyl-containing LTs and LTB_4 (8); furthermore only the cysteinyl-containing LTs are selectively antagonised by the SRS-antagonist FPL-55712-1µg/ml (9).

Assay tissues were prepared as previously described (8). Briefly, male guinea-pigs (Dunkin-Hartley strain 350-400g) were sacrificed by cervical dislocation, lungs and ileum removed and the tissue strips superfused in series with warmed (37°C) oxygenated Tyrode solution (5ml/min) containing a mixture of antagonists: mepyramine (0.1µg/ml), hyoscine (0.1µg/ml), methysergide (0.2µg/ml), phenoxybenzamine (0.1µg/ml) and propranolol (2µg/ml) for increased specificity. Contractions were measured by auxotonic levers (Paton) connected to transducers and visualised on a six-channel pen recorder (Watanabe).

Using the superfusion technique, the actions of both LTB_4 and the cysteinyl-containing LTs are partially mediated via TxA_2 release in guinea-pig lung (10); thus tissue responses to injected samples were compared to standard LTD_4, LTB_4 (1-10pmol) and a TxA_2 mimetic-U44069 (1-3nmol). All extracts were assayed in terms of LTD_4 equivalents (pmol/g) since LTB_4 which only contracts GPP (whereas LTD_4 is also active on GPISM), can produce tachyphylaxis but is equipotent with LTD_4 on this tissue (8).

The use of sensitive tissues, selective receptor

antagonists and the characteristic profile of contractions makes this superfusion biological assay extremely selective for the detection/measurement of LTs.

Radioimmunoassay for LTC_4. Leukotriene C_4 was measured in sputum extracts by RIA using a double antibody technique and an antiserum which also cross-reacted to the extent of 50% with LTD_4 and 8.3% with LTE_4 (11). Cross-reactivity with other eicosanoids, e.g. LTC_4 sulphone, LTB_4, primary PGs, 6-oxo-$PGF_{1\alpha}$, TxB_2 and AA are negligible.

Radioimmunoassay of prostanoids. Samples were assayed for PGE_2, $PGF_{2\alpha}$, 6-oxo-$PGF_{1\alpha}$ and TxB_2 according to the method described by Jose et al (12). Cross-reaction of antiserum to PGE_2 with PGE_1 was 26%; all other cross-reactivities of the four antisera with different COPs (PGE_2, $PGF_{2\alpha}$, PGB_2, PGD_2, 6-0x0-$PGF_{1\alpha}$ and TxB_2) were <3% and with AA <0.06%.

LIQUID SCINTILLATION COUNTING

Radioactivity was estimated in a Packard Tricarb liquid scintillation counter and counting efficiency calculated from a simple channel ratio. All results were converted to disintegrations per minute (d.p.m.). The scintillant used was Aquasol-2 (New England Nuclear).

MATERIALS

The following were used: Leukotriene B_4, C_4, and D_4 (Merck-Frosst Laboratories), FPL-55712 (Fisons), Prostaglandin E_2, $F_{2\alpha}$, D_2, B_2, 6-oxo-$PGF_{1\alpha}$, TxB_2 and U-44069 (The Upjohn Company), mepyramine maleate (May & Baker), hyoscine hydrobromide (BDH), methysergide maleate (Sandoz), phenoxybenzamine and propranolol (ICI).

STATISTICAL METHODS

Results are given as mean + S.E.M. for the number of experiments indicated. The differences between means were considered significant if $P<0.05$ as determined by the unpaired Wilcoxon Rank Sum test (non-parametric).

RESULTS

An equivalent amount of sputum was produced in the two groups of patients (CB=5.4+1.1g wet weight n = 8, B=5.4+0.6g wet weight n = 7).

A bioassay trace showing contraction profiles of LT activity on isolated tissues by a CB sputum extract is represented in Figure 1.

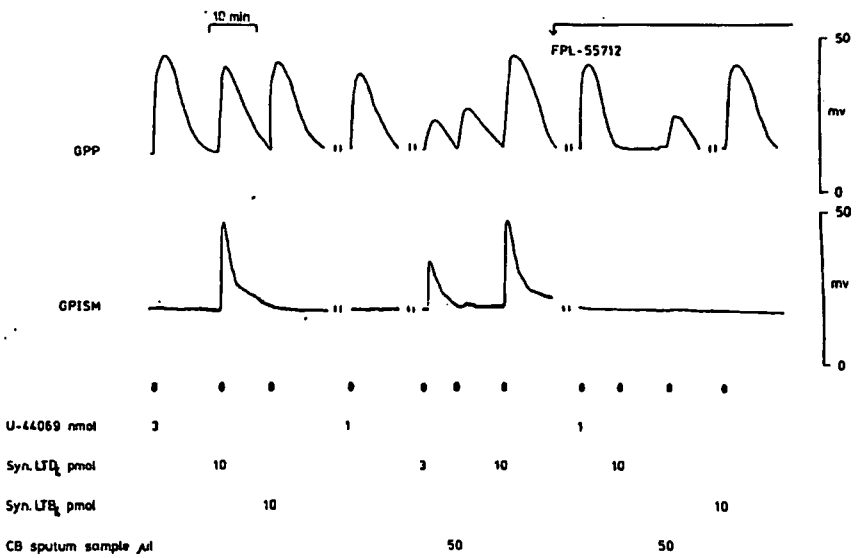

Fig.1. Bioassay of LT-like-activity in sputum of a patients with CB.

The traces illustrate the differential effects of LTs on GPP and GPISM. Synthetic LTB_4 and LTD_4 (10pmol), U-44069 (3nmol) produced contractions of similar height and duration of action (15) mins on GPP. LTB_4 and U-44069 have no effect on GPISM, unlike LTD_4. Injection of a CB sputum extract (50µl) contracted GPP (3-10pmol Eq LTD_4) but produced no effect on GPISM. In the presence of FPL-55712 (1µg/ml), the GPP contraction was almost entirely maintained, whereas the action of synthetic LTD_4 (10pmol) was totally inhibited. Thus LTB_4 accounts for most of the LT activity in CB sputum.

Vertical scale:mv Horizontal scale:10min

For each CB or B sample, contractions of GPP but not GPISM were produced. The overall activity on GPP in sputum extracts of both CB and B patients was 11.9±2.5 pmol EqLTD_4/g, n = 15 (Figure 2).

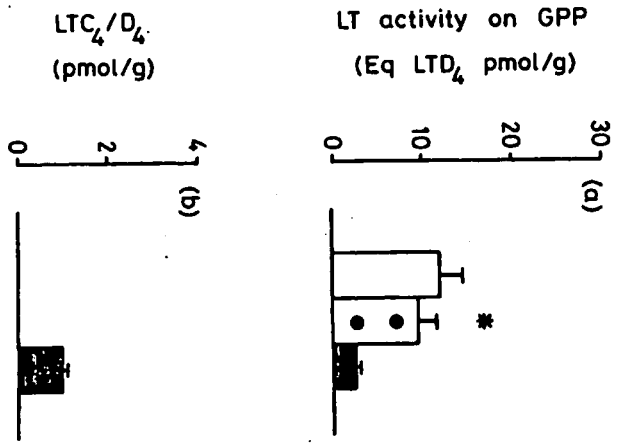

Fig.2. Concentrations of LTs in sputum of patients with either CB and/or B.

(a) Bioassay: The histogram (□) shows LT-like activity on GPP which mainly consists of LTB_4 (●) and also a smaller SRS component (■). LTB_4>SRS by approximately 4-fold. Results are expressed in terms of LTD_4 Eq pmol/g.

(b) Radioimmunoassay: The histogram represents LTC_4/LTD_4 immunoreactivity (pmol/g) which is in broad agreement with the FPL-inhibitable activity measured on GPP.

p = 0.05 compared with the SRS value.

This activity comprised of a major LTB_4 and a relatively minor SRS component which could be inhibited by FPL-55712-1μg/ml (LTB_4 = 9.6±2.0, SRS = 2.3±0.4pmol EqLTD_4/g, n = 15, p = 0.05, Figure 2). The FPL-inhibitable contractions on GPP (represent selective antagonism of cysteinyl-containing LTs) could not be measured on GPISM but were detected by RIA (LTC_4/D_4 immunoreactivity = 1.0±0.4pmol/g, n = 15). The results obtained from these methods are in broad agreement (Figure 2).

Prostanoid concentrations measured by RIA are shown in Figure 3.

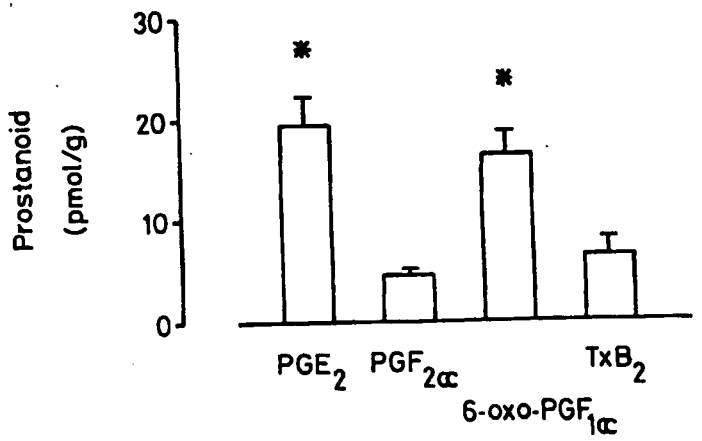

Fig.3. Radioimmunoassay of Ps in sputum of patients with either CB and/or B.

The histogram represents P immunoreactivity for PGE_2, $PGF_{2\alpha}$, 6-oxo-$PGF_{1\alpha}$ and TxB_2 (pmol/g). In CB/B sputum extracts, P synthesis is directed towards the vasodilator rather than vasoconstrictor compounds.

p = 0.05 compared with the value for $PGF_{2\alpha}$ or TxB_2.

All CB/B samples showed COP-immunoreactivity: PGE_2 = 19.3±2.8, $PGF_{2\alpha}$ = 4.5±0.6, PGI_2 as represented by its relatively stable metabolite 6-oxo-$PGF_{1\alpha}$ = 16.4±2.1 and TxA_2 reflected by the hydrolysis product TxB_2 = 6.3±1.9 pmol/g, n = 15.

DISCUSSION

We have detected LTs and Ps in the sputum of patients with CB and/or B.

Since the application of bronchography or computerised axial tomography was felt to be clinically unjustifiable, it was difficult to differentiate CB from B with absolute reliability. We have, therefore, pooled the results from both groups in the analysis of the data.

Although partially purified samples were assayed, the superfusion bioassay and RIA techniques used in this study permit the detection of eicosanoids with a high degree of certainty and accuracy (see methods 8, 11, 13). Furthermore, the recovery of radiolabelled LTs and Ps using alcohol and octadecylsilyl methods produces less than an 8% variation in extraction efficiency (14, 15).

Our results show that in sputum extracts obtained from CB/B patients, LTB_4 is present in significantly higher levels than the cysteinyl-containing LTs (LTC_4/LTD_4). A possible explanation for these differences may stem from the nature of the inflamatory cells, particularly neutrophils which predominate in CB/B sputum and readily synthesize LTB_4 (16). Sputum eosinophilia has been reported as a feature of CB (17) and human eosinophils predominantly secrete LTC_4 (18) which suggests that these cells may be a possible source of cysteinyl-containing LTs. Nevertheless, the sputum concentration of arylsulphatase IIB, which inactivates SRSs, may also contribute to decreased levels of LTC_4/LTD_4, particularly in the bronchiectatic patients (19, 20).

Differences in the P content of CB/B sputum were also apparent. The pathway of AA oxidation via cyclo-oxygenase appears to be directed towards the vasodilator (PGE_2, PGI_2) rather than vasoconstrictor ($PGF_{2\alpha}$, TxA_2) products. The bias towards the formation of vasodilator Ps has also been observed in cystic fibrosis (5) and is of interest since in some animal models interaction of vasodilator Ps with LTs induces vascular permeability changes and oedema formation (21).

Although histological analysis of sample extracts was not undertaken, it is well documented that sputum obtained from CB/B patients contains many different inflammatory cells (4, 6, 17) which contain the necessary enzymatic machinery for the synthesis of 5-lipoxygenase and cyclo-oxygenase products (1, 2).

The mechanism of eicosanoid release is not fully understood in these pulmonary disorders. However, for CB at least, elevated sputum but not serum IgE concentrations have been reported suggesting a local Type I hypersensitivity reaction (17). Although it has been proposed that CB and B patients do produce a hyperimmune response, there is no significant increase in the prevalence of atopy in these subjects (17).

LTs and Ps have a wide range of actions including stimulation of mucus secretion (22), contraction of isolated bronchial smooth muscle (23), bronchoconstriction (24) and oedema formation (21). Furthermore, LTB_4 is a potent chemotatic/kinetic and aggregating agent for leukocytes (16, 25).

The quantitation of eicosanoids from HPLC-purified samples has not been undertaken in this study. Nevertheless, the presence of LTs and Ps in CB/B sputum in amounts likely to produce biological effects merits their consideration as possible mediators in these diseases.

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PROSTACYCLIN AS NEUROMODULATOR IN THE SYMPATHETICALLY STIMULATED RABBIT HEART

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ABSTRACT

Prostaglandin E_2 (PGE_2) has previously been shown to inhibit sympathetic neurotransmission in different organs and species. Based on this inhibitory effect and on its reversal by cyclo-oxygenase inhibitors, PGE_2 has been claimed to be a physiological modulator of in vivo release of norepinephrine (NE) from sympathetic nerves. It is now recognized that prostacyclin (PGI_2) is the main cyclo-oxygenase product in the heart. We therefore addressed the question whether PGI_2, within the same preparation, is formed in increased amounts during sympathetic nerve stimulation and has neuromodulatory activity.

The effluent from isolated rabbit hearts subjected to sympathetic nerve stimulation or to infusion of NE or adenosine (ADO) was collected, and its content of PGE_2 and 6-keto-$PGF_{1\alpha}$ (dehydration product of PGI_2) was analyzed using gas chromatography/mass spectrometry, operated in the negative ion/chemical ionization mode. Other hearts were infused with PGI_2 and nerve stimulation induced outflow of endogenous NE into the effluent was analyzed using HPLC with electrochemical detection. Nerve stimulation at 5 or 10 Hz (before but not after adrenergic receptor blockade), as well as infusion of NE (10^{-6}-10^{-5}M) or ADO (10^{-4}M) increased the cardiac outflow of 6-keto-$PGF_{1\alpha}$. Basal and nerve stimulation induced efflux of 6-keto-$PGF_{1\alpha}$ was approximately 5 times higher than the corresponding efflux of PGE_2. PGI_2 dose-dependently inhibited the outflow of NE from sympathetically stimulated hearts, the inhibition at 10^{-6}M being approximately 40%.

On the basis of these observations we propose that PGI_2 is a more likely candidate than PGE_2 as a potential modulator of neurotransmission in cardiac tissue in vivo.

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NEPHROLOGY FORUM

Acute renal failure and the sepsis syndrome

Exhibit 8

Principal discussant: NESTOR SCHOR

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CASE PRESENTATION

A 56-year-old man was admitted to the hospital with acute suppurative appendicitis. A laparotomy was performed, and he developed sepsis after the surgery. After seven days he was transferred to the intensive care unit of the Escola Paulista de Medicina with necrotizing fasciitis (Fournier's syndrome), respiratory distress, and increased levels of serum creatinine (2 mg/dL). At admission he had fever (38°C), normal blood pressure (140/90 mm Hg), normal urinary flow with a FE_{Na} of 0.9%, and was under mechanical ventilation. That same day an exploratory laparotomy revealed significant necrosis of the abdominal wall and an intracavitary infection. A peritoneostomy was performed.

Hemodynamic monitoring with a Swan-Ganz catheter revealed normal pulmonary capillary wedge pressure (16 mm Hg) and a septic profile (high cardiac index and low systemic vascular resistance index). Despite a normal urinary output of 70 to 90 mL/h, the serum creatinine gradually increased to 5 mg/dL over the following 6 days. During this period he underwent two abdominal surgeries and daily hyperbaric oxygen therapy in a 2.5 atmosphere chamber, while receiving metronidazole, vancomycin, and a third-generation cephalosporin. No aminoglycoside antibiotic was administered. No bacteria or fungi were identified in blood, urine, or even in the abdominal secretions.

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Key words: lipopolysaccharide, endotoxin, endothelin, platelet activating factor, tumor necrosis factor, nitric oxide, ischemic preconditioning, hyperkalemia.

On day 6 (ATN-Liãno score of 0.838; APACHE II of 23; APACHE II risk of 45%), continuous veno-venous hemofiltration and dialysis (CVVHD) was initiated and maintained for 3 days. Intermittent daily hemodialysis using a synthetic (polysulfone) membrane was then introduced. Fourteen sessions of hemodialysis were performed, and the blood urea and serum creatinine levels were kept below 180 and 5 mg/dL, respectively. Only one episode of hyperkalemia (5.8 mEq/L) with acidosis (pH of 7.21 and plasma HCO_3^- of 14 mEq/L) was observed. Total parenteral nutrition was administered throughout this period. His urinary output dropped to 20 to 30 mL/h after the introduction of hemodialysis and began to increase (40 to 50 mL/h) after 10 days. During that period, weaning from the ventilator was started and the hyperbaric oxygen therapy was discontinued.

By day 26 after admission, his urinary output had reached 110 mL/h and the serum creatinine had progressively decreased. He was discharged from the hospital on day 32 with a serum creatinine of 1.2 mg/dL and a creatinine clearance of 70 mL/min.

DISCUSSION

DR. NESTOR SCHOR (*Dean, Postgraduate Programs and Research; and Professor of Medicine, Disciplina de Nefrologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, Brasil*): In the present patient, the diagnosis of sepsis was readily made by the presence of one major criterion, necrotizing fasciitis (Fournier's gangrene), and two minor criteria: respiratory distress and fever. Acute renal failure (ARF), as often occurs, complicated the clinical condition [1].

Hemodynamic monitoring revealed a hyperdynamic pattern, with low systemic vascular resistance and high cardiac output, both of which are characteristic of septic shock. The pulmonary capillary wedge pressure (PCWP) was normal at 16 mm Hg, although PCWP can be a misleading index of left-ventricular pre-load (defined as volume). But sequential measurements of end-diastolic volumes are not routinely available. Importantly, diastolic dysfunction, a common feature of septic shock, is attributed, at least in part, to an interstitial myocarditis that is a classic acute inflammatory response characterized by a polymorphonuclear cell infiltration and varying amounts of cellular tissue necrosis [2]. Therefore, the search for an ideal indicator of myocardial dysfunction in sepsis is an ongoing and important issue. Troponin 1 has recently

been recognized as a promising tool for the identification of myocardial injury in sepsis [3].

Sepsis, especially septic shock, is one of the main causes of ARF. The prevalence of ARF in sepsis ranges from 9% to 40% [4]. Liãno et al recently presented data from the Madrid Acute Renal Failure Study Group [5] indicating that sepsis caused acute tubular necrosis in 35% of patients in an intensive care unit (ICU) and 27% of non-ICU patients. In a French multicenter study, ARF was related to sepsis in 48% of the patients [6]. Also, the mortality rate was higher in septic patients with ARF than in nonseptic patients (73% vs. 45%). In a prospective study including a large number of patients with sepsis and septic shock, the incidence of ARF was 19% in sepsis, 23% in severe sepsis, and 51% in septic shock [7]. Using data from the General Hospital of the University of São Paulo, Burdmann et al reported that 35% of the cases of ARF resulted from sepsis, 33% from hypovolemia, 32% from congestive heart failure, and 27% from nephrotoxic agents (mainly antibiotics) [1]. The mortality rate reached 50% in patients with the multiple organ dysfunction syndrome (MODS) in the ICU, compared with a 7% mortality rate in patients in a general hospital [1]. The primary mediators of the sepsis syndrome and its deadly complications are the endotoxins.

Endotoxins are lipopolysaccharides (LPS) of the outer membrane of gram-negative bacteria. The effects of endotoxin depend on the equilibrium between the cellular mediators released and their natural antagonists. The effects also are influenced by the nature of the endotoxin and by genetic factors. The marked heterogeneity of LPS is responsible for the diversity of their biologic properties, for instance, the activation of complement, cytokine production, toxicity, and resistance to antibiotics. Although LPS of *E. coli* are the most frequent and important endotoxins studied, other LPS from gram-negative bacteria also might participate in the septic syndrome.

Our understanding of the molecular events involved in the recognition of LPS by target cells is incomplete, but a key role for the CD14 receptor was established by the finding that CD14-deficient mice are insensitive to the effects of LPS [8]. Studies on the interaction of LPS and other inflammatory mediators indicated that the first step in the cascade is the binding of LPS to a specific soluble protein, the LPS-binding protein (LBP) (Fig. 1). The LPS-LBP complex interacts with the glycosylphosphatidylinositol (GPI)-anchored CD14 receptor in the membrane (mCD14) of polymorphonuclear neutrophil (PMN) cells, macrophages, and other cells such as mesangial cells [9]. The interaction of the LPS-LBP complex with CD14 leads to phosphorylation of the complex formed by the nuclear factor-κB (NF-κB), a member of the Rel family of transcription factors (p50, p56, and p65, among others), and its inhibitor (I-NF) via an IκB kinase (IκK) [10]. The subsequent release of NF-κB from IκB results in the translocation of NF-κB to the nucleus, where it binds to specific sequences in the promoter regions of target genes. A large number of genes appear to be targets of NF-κB. These genes are responsible for the coding and transcription of several cytokines and chemokines, for instance, tumor necrosis factor-α (TNFα), platelet activating factor (PAF), leukotrienes/prostaglandins (LT/PGs), interferon γ, interleukin (IL)-1, IL-6, IL-8, and IL-12. In the systemic circulation, these molecules can cause hemodynamic imbalance and cell/organ dysfunction and can lead to apoptosis, necrosis, and death.

Recently it was shown that receptors of the "toll family" play a major role in the induction of immune and inflammatory responses [11]. The first receptor of the toll family was identified in Drosophila as a component of a signaling pathway that controls polarity in fly embryos. The sequence of the *toll* gene revealed that it encodes a transmembrane protein that has a cytoplasmic domain similar to the mammalian interleukin-1–receptor; the transmembrane protein leads to the activation of transcription factors of the NF-κB family. Homologs of Drosophila toll are referred to as toll-like receptors (TLRs). The first human TLR to be characterized, TLR4, activates the NF-κB signaling pathway. Another protein, the MD-2, is required for TLR4-mediated recognition of LPS, forming a recognition complex of CD14, TLR4, and MD-2. After this complex is activated, it recruits the adapter protein MyD88, which is associated with the serine-threonine protein kinase interleukin-1–receptor–associated kinase (IRAK). In turn, IRAK is phosphorylated and associated with the tumor necrosis factor–associated factor 6 (TRAF-6) adapter protein. The oligomerization of TRAF-6 activates a member of the mitogen-activated protein kinase kinase kinase family (MAP3K) that activates the IκB kinase 1 (IκK1) and 2 (IκK2) [11]. These kinases phosphorylate IκB on serine residues and release NF-κB, which moves to the nucleus.

The mCD14 molecule can be released from the GPI in the membrane and circulates in the serum (sCD14; Fig. 1). The sCD14 linked with LPS stimulates endothelial and epithelial cells (including renal tubular cells). The cells not only release cytokines and chemokines, but also VCAM, ICAM, selectins, and MCP-1 (monocyte chemoattractant protein-1) [12], which are responsible for the adhesion of PMN cells and monocytes to endothelial cells. These mediators also increase vessel permeability, which leads to contraction of intravascular volume and hypotension. The alteration caused by these adhesion molecules on the tubular epithelium leads to cell detachment, resulting in tubular obstruction and cellular necrosis. Data obtained in the ischemia/reperfusion model in the mouse showed high levels of renal ICAM-1 mRNA, and increased systemic levels of IL-1 and TNFα one hour after ischemia/reperfusion. Protection of the

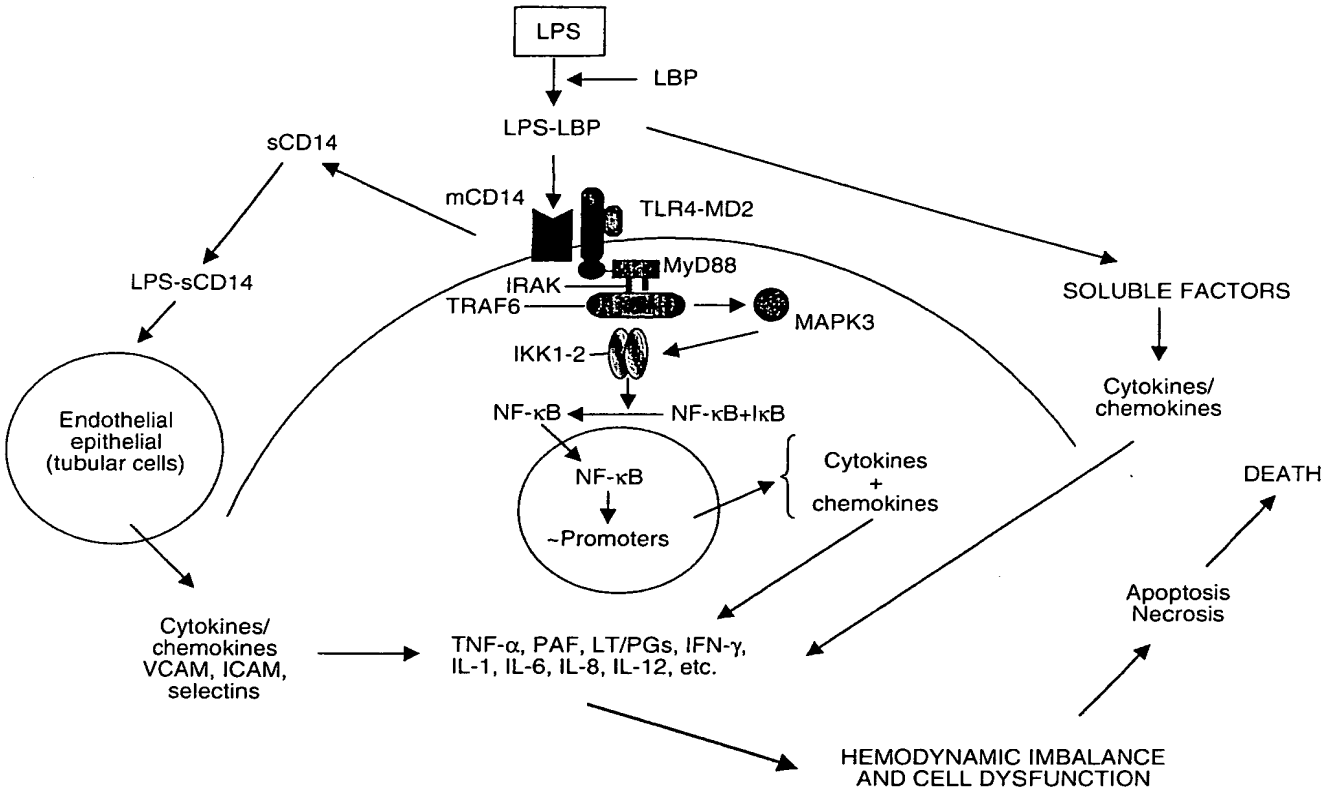

Fig. 1. Mediators of cellular origin (see text for abbreviations).

glomerular filtration rate (GFR) was observed in ICAM-1 knockout mice as well as in neutrophil-depleted mice. However, treatment of the latter with anti-ICAM-1 antibodies conferred no additional protection. Thus, ICAM-1 is a possible mediator of ischemic acute renal injury, acting via the potentiation of neutrophil-endothelial cell interaction [13]. The activation of NF-κB in peripheral blood mononuclear cells of patients with sepsis is increased in patients who die when compared with survivors [14].

Lipopolysaccharide also can activate soluble factors (that is, cytokines and chemokines in circulation (Fig. 1) and initiate the septic cascade. The same overall mechanism, operative in PMNs, also is observed in mesangial cells. Many factors block NF-κB activation, such as glucocorticoids, cyclosporine, angiotensin-converting enzyme inhibitor (ACEI), lovastatin-like drugs, etc. [15]. It is possible that the effect of ACEI in delaying the progression of sepsis-induced acute renal failure is caused by a reduction of NF-κB formation. The presence of an amplifying loop, given a positive feedback further producing inflammatory and other molecules, could explain the maintenance of a low GFR via contraction of mesangial cells and interference with other glomerular structures despite the shut-off of the initial pathophysiologic stimulus.

Pathophysiologic mediators

The clinical forms of acute renal failure vary from acute tubular necrosis (ATN) to bilateral cortical necrosis. A large body of evidence from experimental models and human endotoxemia studies suggests that various inflammatory mediators and neuro-endocrine mechanisms are involved in the pathogenesis of ARF in sepsis. The interplay of these pathways results in severe renal hypoperfusion, a major factor in the development of ARF. However, non-hemodynamic responses to endotoxin contribute importantly to the acute renal dysfunction.

Lugon and co-workers in our laboratory observed a decrease in both total and single-nephron GFR in endotoxemic rats that was mediated by high renal vascular resistance (RVR) and decreased renal plasma flow (RPF; Fig. 2) [16]. The impressive increase in RVR was a consequence of a high afferent arteriolar resistance. The elegant studies by Cohen and co-workers showed that LPS alone, in a serum-free, artificially perfused rat kidney, did not modify GFR or tubular function, and suggested that extrarenal mediators are required [17]. In a similar

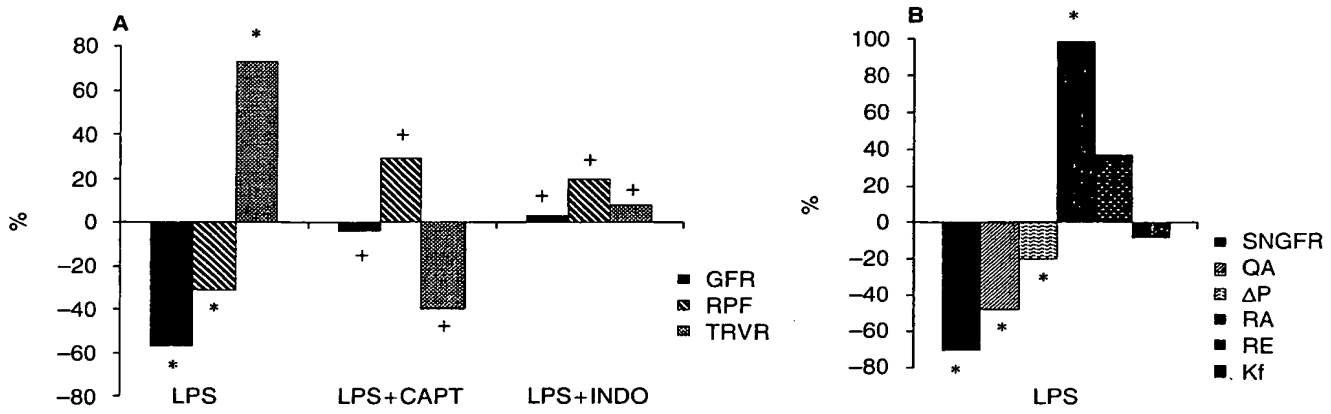

Fig. 2. Effects of lipopolysaccharides (LPS) on renal (*A*) and glomerular (*B*) hemodynamics: Role of angiotensin and prostaglandin systems. Abbreviations are: CAPT, captopril; INDO, indomethacin; GFR, glomerular filtration rate; RPF, renal plasma flow; TRVR, total renal vascular resistance; SNGFR, single-nephron glomerular filtration rate; QA, glomerular plasma flow; ΔP, transglomerular hydraulic pressure; RA, afferent, and RE, efferent, arteriolar resistances; Kf, glomerular ultrafiltration coefficient. $P < 0.05$ after versus before LPS infusion; $+P < 0.05$ versus LPS alone.

experimental model of isolated perfused kidney, Linas and colleagues observed that the simultaneous perfusion with LPS and PMN cells (previously activated with LPS and formyl peptides) caused a striking decrease in GFR, and suggested a necessary interaction for the induction of ARF [18]. They also suggested that PMN cell activation releases both oxygen metabolites and elastase. Here I will review briefly the most important mediators of the hemodynamic response.

Tumor necrosis factor (TNF), a pleiotropic cytokine, mediates a broad spectrum of host responses to endotoxemia. It is released from circulating and resident (mesangial) cells following stimulation by LPS [19, 20]. Levels of TNF are elevated in patients with septic shock and are correlated with the severity of sepsis and mortality rate [21]. In turn, TNF stimulates a cascade of vasoactive and inflammatory mediators that participate in the septic syndrome [4] and in the renal response to LPS. Local release of TNF from mesangial cells following stimulation by LPS induces apoptotic death of glomerular endothelial cells [20]. Passive immunization to TNF protects against the renal effects of LPS in rhesus monkeys [22].

Platelet activating factor (PAF) is another mediator released by glomerular endothelial and mesangial cells in response to LPS [4]. The glomerular hemodynamic effects of PAF include increases of both afferent and efferent arteriolar resistances that cause reduction of the glomerular plasma flow and intraglomerular hydraulic pressure; these changes, coupled with an associated decrease in Kf, produce a substantial decline of GFR [23]. The levels of PAF in blood and urine are elevated in septic patients and correlate with the severity of ARF [24]. An impressive reduction in mortality is seen in animal models of sepsis treated with anti-PAF antibody, suggesting an important role of PAF in septic shock [25].

Endothelin-1 (ET), a peptide with highly potent vasoconstrictor activity, reduces GFR and RPF as a consequence of its effects on afferent and efferent glomerular arterioles. Tumor necrosis factor, vasopressin, angiotensin II, and PAF are among the factors that stimulate the release of ET-1 [4]. In addition to circulating ET-1, locally synthesized ET-1 in the kidney by various types of renal cells, including mesangial and endothelial cells, acts as an autocrine and paracrine factor. In an elegant protocol, Kon and Badr showed that the intrarenal administration of anti-ET-1 antibody blunted the renal hypofiltration observed during a systemic infusion of LPS [26].

The inducible nitric oxide (NO) synthase (iNOS), through NO generation, has been implicated in the hemodynamic alterations observed in sepsis, mainly induction of vasodilation [4]. The expression of iNOS is induced by TNF and IL-1 in macrophages, vascular smooth muscle, and mesangial cells. On the other hand, local NO regulates efferent arteriolar tone, thus influencing the maintenance of renal hemodynamics. Nitric oxide inhibition during endotoxic injury increases proteinuria, decreases GFR, and leads to a marked glomerular thrombosis, suggesting an important protective role of this mediator in septic ARF [27]. Weight and colleagues have observed a bimodal response to NOS inhibition in the ischemia-reperfusion model, with an early amelioration but a late exacerbation of the oxidative damage; these data suggest that although the early burst of NO can have a cytotoxic action, NO might exert an overall cytoprotective effect [28]. Acute L-arginine administration is beneficial in in-vivo renal ischemia but harmful in isolated tubules made hypoxic and reperfused [29].

Oral supplementation or intravenous infusion of L-arginine supports a protective effect of NO on renal function in a number of nephrotoxic models of ARF such as those induced by contrast media [30], cyclosporine A (CsA) [31], glycerol [32], uranyl nitrate [33], and gentamicin [34].

During ischemia, antisense oligonucleotides to iNOS, but not the knocking out of the iNOS gene, attenuated the LPS-induced fall in GFR [35]. The latter effect was due to an attenuation of the tubular injury [36] and suggests cytotoxic effects of the NO produced via iNOS during the ARF. Thus, the available data indicate a variable role of NO in ARF, and the ultimate (cumulative) effect will depend on the experimental model, the potency of inhibitor/stimulator of the NO system, and the stage of ARF studied.

Thromboxane A_2 (TxA_2) is the major vasoconstrictor product of the cyclo-oxygenase pathway causing declines in RPF and GFR due to preferential constriction of the afferent arterioles [4]. Lipopolysaccharide is a potent stimulator of TxA_2 synthesis in renal cortex, and various reports have provided evidence for a role of TxA_2-induced vasoconstriction in models of experimental sepsis through the release of PAF [37]. Selective antagonists afford protection against renal ischemia. Data from our laboratory showed that indomethacin, a nonspecific inhibitor of prostaglandins and thromboxanes, had a markedly protective effect on glomerular hemodynamics in endotoxemic rats, suggesting that TxA_2 might play an important role in LPS-induced ARF (Fig. 2) [16].

Leukotrienes (LTs) are also implicated in the pathophysiology of sepsis-induced ARF, as they are highly potent vasoconstrictor eicosanoids [4]. These compounds (LTC_4, LTD_4, and LTE_4) are produced by the 5-lipoxygenase enzyme pathway and are related to the slow-reacting substances of anaphylaxis. Their vasoconstrictive property causes a reduction of RPF and GFR mainly due to pre-glomerular vasoconstriction and associated reduction in Kf, the latter likely indicating mesangial cell contraction [38]. Leukotriene levels are increased during sepsis, and use of LT antagonists minimizes the effects of intravenous LPS administration, including the acute renal dysfunction [37].

A number of other important humoral vasoactive mediators also are implicated in septic ARF, including the renin-angiotensin system (RAS), the kallikrein-kinin system, atrial natriuretic factor, IL-1, adenosine, and catecholamines. The role of the RAS on LPS-induced ARF is indicated in Figure 2, which shows that captopril reduced the adverse effects of LPS on renal hemodynamics.

Beyond the hemodynamic adversity, the aforementioned mediators also contribute to the acute renal dysfunction by non-hemodynamic responses to endotoxin, including activation of the complement system, and of the coagulation and fibrinolysis cascade, thereby causing intrarenal coagulation [39]. Moreover, neutrophil activation, which is high in patients with ARF and sepsis, resulted in reduction of GFR [40]. As I mentioned, in the isolated perfused kidney, the GFR-reducing effect of the LPS was only evident in the presence of endotoxin-activated neutrophils [18].

In summary, by interacting with circulating and resident cell types, such as monocytes/macrophages and endothelial, PMN, and mesangial cells, endotoxin stimulates the release of inflammatory cytokines and other vasoactive substances. These mediators coupled with neuroendocrine factors and the coagulation/fibrinolysis cascades, which are activated by endotoxin, lead to profound alterations of renal function in sepsis and ARF.

Because the various cytokines and vasoactive compounds discussed earlier induce tissue ischemia, it is interesting to examine preconditioning situations related to ARF. Ischemic preconditioning (IPC) is a general term used to describe maneuvers that increase tolerance of a tissue to a prolonged, potentially lethal, period of ischemia. Although the beneficial effects of IPC were first observed in the myocardium, it is now clear that IPC also protects the brain, liver, lung, and several other organs, including the kidney. Although the different steps involved in this phenomenon are far from fully elucidated, the currently favored hypothesis is that IPC initiates, via receptor stimulation, activation of phospholipase C and/or D, production of the second messenger diacylglycerol (DAG), and the resultant activation and subcellular redistribution of one or more of the known isoforms of protein kinase C (PKC) to the membrane; the latter events potentially trigger a kinase cascade that ultimately culminates in the phosphorylation of the final effector agents [41].

Differential display polymerase chain reaction and differential cDNA library screening techniques have been used to identify differentially expressed genes after renal blood flow cessation of 30 to 60 minutes. These techniques have led to the identification of genes that code for proteins apparently essential for either renal tubular cell death, such as Siva (a pro-apoptotic protein), or for cell regeneration, such as calcyclin and the receptor for activated C kinase (RACK1) [reviewed in 42]. Using an IPC renal model, Gomes, Cancherini, and Rebouças suggested that the mechanisms of IPC must include up-regulation of proteins involved in cytoskeleton organization, such as tropomyosin 4, caldesmon, actin depolymerizing factor, or proteins related to both preservation and recovery of the functional folding of these proteins, such as HSP70, a heat shock protein, and CCTα, a member of the chaperone family, whose mRNA levels were increased by approximately 50% (unpublished data). They also observed an up-regulation of proteins related to oxidative phosphorylation, such as NADH-ubiquinone

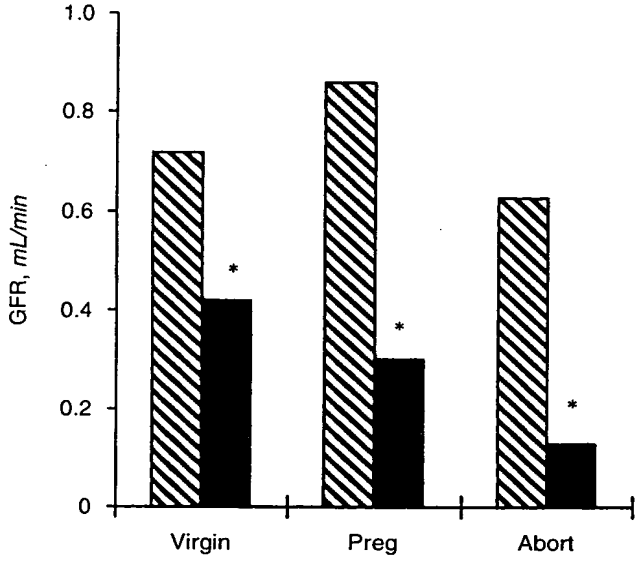

Fig. 3. Total glomerular filtration rate (GFR) in virgin, pregnancy (Preg), and abortion (Abort) groups before and after infusion of lipopolysaccharide (LPS). $*P < 0.05$ verus before LPS.

reductase and cytochrome c oxydase subunit II, which might be important for fast recovery or preservation of the mitochondrial complex I after ischemic insult.

An important event related to both preconditioning and recovery is the synthesis of heat shock proteins (HSP; stress proteins) in response to a mild stress, a phenomenon that allows cells to adapt to gradual changes in their environment and to survive under potentially lethal conditions [43, 44]. Heat shock proteins alone or in combination with chaperones are involved in several cellular processes, such as correction of the folding and assembling of intracellular proteins, transport and degradation of proteins, as well as preservation and restructuring of the cytoskeleton [44]. The presence of various HSP (for example, HSP70, HSP72, HSP25/27) with a specific distribution pattern along the nephron segments, both in mesangial and epithelial cells, indicates a potential role for HSP not only in initiation and maintenance but also in recovery from injury, including endotoxic insult [44].

Using a model of abortion induced by RU-486 with or without LPS administration, Boim and coworkers in our laboratory showed that LPS was more effective in decreasing GFR in pregnant than in virgin rats (Fig. 3) [45]. However, after the induction of abortion, the LPS effect increased. This finding suggested a sensitization to the toxic effects of LPS in this clinical situation. This potentiation might explain the high morbidity and mortality rates of septic abortions.

Prognosis

In the clinical setting, prognostic score indices often have been used in the prediction of outcomes of ARF patients. These indices, used mainly in ICUs, are useful tools for quality control, allocation of resources in the ICU, comparison of different units, and adequate randomization in clinical trials. In recent years, the APACHE II score has been the most widely used prognostic score index in the ICU. Although it has been validated only in ICU patients, it has been used for non-ICU patients with ARF [46]. The APACHE II score has been used to randomize patients with ARF at the time of initiation of dialysis in clinical trials as well [47]. We investigated the differences between ICU and non-ICU patients with ARF, and sought to determine whether the APACHE II score can be used as a prognostic score index for non-ICU patients (abstract, Fernandes et al, *J Am Soc Nephrol* 10:140A, 1999). We compared the performance of the APACHE II score with that of the acute tubular necrosis-individual severity score (ATN-ISS), a specific score for the population with ARF [48]. Data were collected from 205 ARF patients at the Hospital São Paulo, a university-based, non-profit, tertiary hospital. APACHE II scores were calculated at the time of hospital admission (AP-1), at referral to the nephrologist (AP-2), and at initiation of dialysis (AP-3). The ATN-ISS score was obtained only once, when the patient was referred to the nephrologist. Data were similar between ICU and non-ICU patients for AP-1, AP-2, and ATN-ISS. Thus, the APACHE II score collected at hospital admission or at the time of referral to the nephrologist and the ATN-ISS score can be used as a severity-of-illness score for non-ICU patients (Fig. 4). The discrimination capacity of APACHE II and ATN-ISS (area under the ROC curves in Fig. 4) was similar. Thus, when using APACHE II, the chance for detecting those patients who were likely to die was similar for both ICU (AUC=0.66) and non-ICU (AUC=0.78) patients. Therefore, these results also support the utilization of APACHE II for non-ICU patients. The time of collection of data for the APACHE II score was investigated, and scores obtained at the time of referral to the nephrologist were better than scores obtained at hospital admission or at the initiation of dialysis. Thus, ARF per se seems to be an important determinant of prognosis. The ATN-ISS score was a better predictor of death than was the nonspecific APACHE II score. In another Brazilian study, Batista evaluated different prognostic indices, including the ATN-ISS score, the APACHE II score, the long injury score (LIS), and the number of organ failure (NOF) score, in ARF patients at the ICU of São Rafael Hospital, a teaching hospital in the city of Salvador [49]. In contrast with the study at Hospital São Paulo, the APACHE II and ATN-ISS scores showed similar discriminative power. This fact illustrates the need for studying the discrimination and calibration of these prognostic scores for each hospital.

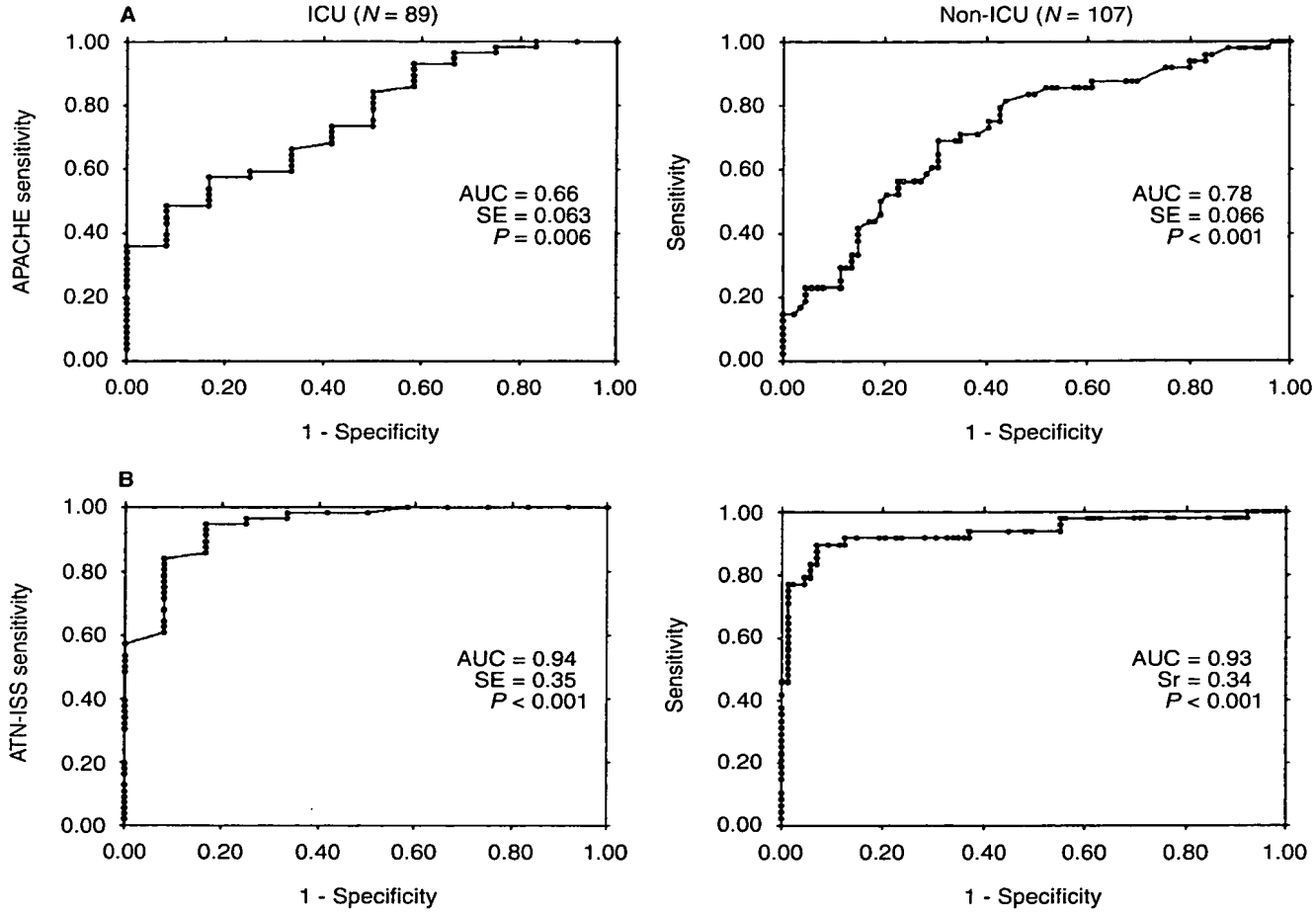

Fig. 4. Discrimination analysis (ROC curves) of APACHE II and ATN-ISS indices obtained at the time of referral to the nephrologist of ICU and non-ICU patients. AUC, area under the curve.

Clinical issues

Trials. Only a few multicenter clinical trials have attempted to change the high mortality rate observed in patients with ARF and the septic syndrome. One of them was based on the observation that insulin-like growth factor-I (IGF-I) increased RPF and GFR in normal rats, in normal adult humans, and in patients with chronic renal failure [50]. Also, IGF-I buttressed the recovery of GFR, healing the affected nephrons and reducing the mortality rate in animals after ischemic, toxic, or endotoxic ARF [51]. In a multicenter trial, with a prospective, double-blind, randomized design, patients with ARF of all types received rhIGF-I versus placebo [52]. Baseline values of GFR, urinary volume, BUN, serum creatinine, and albumin were similar between groups, as was the need for dialysis. Thirty-seven percent of the patients receiving rhIGF-I and 35% of those receiving placebo had septic ARF. Treatment with IGF-I failed to change recovery and mortality rates. A negative result also was observed with ANF (anaritide) in another trial of ARF patients [53]. These results emphasize the importance of the development of multitargeted therapeutic strategies because of the multifactorial nature of the pathophysiology of sepsis-associated ARF.

Hyperkalemia. In today's patient, the only episode of hyperkalemia was largely ascribed to reduced potassium excretion, a frequent indication for initiating dialysis. The decrease in potassium excretion observed during ARF is predominantly caused by a low tubular flow rate due to a reduction in GFR and accompanying epithelial cell damage. Under normal conditions, tubular epithelial cells respond to variations in serum levels of potassium and/or plasma aldosterone with adequate potassium secretion in cortical collecting ducts (CCD), increasing or reducing renal potassium excretion. However, during renal insufficiency, even the viable cells might not be able to secrete

potassium properly. The density and the activity of potassium-secreting channels in the apical membrane of CCD cells might be involved in this deficiency. Rabb and coworkers showed that the changes in potassium handling in ischemic ARF are associated, at least in part, with a suppression of the potassium secretory channel (ROMK1) mRNA [54]. On the other hand, a reduction in proximal sodium reabsorption can increase sodium delivery and potassium secretion in the CCD. The deficiency in sodium reabsorption along the nephron is related to several sodium transporter mechanisms, including the redistribution of Na/K/ATPase, mainly in proximal tubules, and the down-regulation of sodium transporters, including Na/K/ATPase, Na/H exchanger (NHE3), Na/K/2Cl cotransporter, and Na/Cl cotransporter [55]. Although the high sodium concentration in the tubular fluid and/or the high levels of aldosterone would tend to augment potassium secretion in the CCD, this process is impeded by the suppression of the ROMK1 channels. Moreover, the colonic K/H/ATPase mRNA in the distal nephron is overexpressed during ischemic/reperfusion injury [56]; the resultant increased hydrogen secretion prevents bicarbonate wasting, but the increased potassium reabsorption contributes to the hyperkalemia.

Although hyperkalemia is a life-threatening complication of ARF, safe levels of serum potassium are often achieved in ARF, even with extremely low GFR. Redistribution of potassium into cells is one potential adaptive mechanism for reducing serum potassium levels. Moreover, intestinal potassium excretion can substantially increase in patients with renal insufficiency. The channel-inducing factor (CHIF), a molecule expressed in distal parts of the nephron and intestine and regulated by potassium intake and aldosterone, respectively, might play a role in potassium homeostasis in ARF. In fact, expression of CHIF mRNA is dramatically decreased in the kidney and increased in the colon in a model of acute tubular necrosis induced by glycerol injection [57]. Thus, the deficiency in renal potassium excretion during ARF is a result of a complex interaction among several factors. The level of serum potassium concentration depends on the extent to which each of these factors is altered in a particular patient as well as on changes in redistribution of potassium into cells and intestinal potassium excretion.

Dialysis

Because the risk of death in patients with ARF increases with the need for dialytic support, many issues regarding the use of renal replacement therapy have been investigated. These include timing of initiation, modality, type of dialyzer membrane, and dose of dialysis. The type of dialyzer membrane used during intermittent hemodialysis (IHD) has received the most attention.

Several methods of dialysis are available for renal replacement therapy (RRT). Continuous RRT (CRRT), the most frequently used approach in the intensive care unit, is potentially indicated for the removal of inflammatory mediators; most studies in critically ill patients with sepsis and ARF have demonstrated the presence of such mediators in the ultrafiltrate [58]. However, the process itself involves induction of cytokines and complement activation, so elimination of natural inhibitors of pro-inflammatory substances also can occur. To address this issue, Vriese and colleagues performed continuous venovenous hemofiltration (CVVH) with an AN69 membrane in septic patients with ARF [59]. They observed a decline in some cytokines during the first hour of hemofiltration, but a partial recovery occurred after 12 hours. Their study suggested a beneficial effect of CVVH, especially if the membrane is frequently replaced and a high ultrafiltration rate is used.

Cardiovascular instability is the most common complication of dialysis, especially in critically ill patients who have established ARF. In this setting, intermittent hemodialysis increases the risk of hemodynamic instability. However, the slow nature of CRRT allows for a slow rate of ultrafiltration with a lower impact on blood pressure. In general, CRRT is indicated for hemodynamically unstable and fluid-overloaded patients; intermittent hemodialysis is better for patients who are hemodynamically stable. An individualized approach should be used for each patient to optimize management of ARF. Despite significant advances in technology, the effect of CRRT on overall patient outcome remains unclear. No study has showed an increased rate of survival with CRRT as opposed to HD in patients with ARF [60].

In patients with chronic renal failure, the use of dialyzers with unsubstituted cellulose membranes results in complement and leukocyte activation with the generation and release of cytokines by peripheral blood mononuclear cells (PBMCs) [61] and the production of reactive oxygen species by neutrophils [40]. It thus has been hypothesized that in the setting of ARF, production of these inflammatory mediators during dialysis with unsubstituted cellulose membranes leads to a worsening of the inflammation within the kidney, a prolonged need for dialysis, and a worse outcome.

Despite the fact that the molecular basis for the possible benefit of more biocompatible membranes has not been studied in humans, a number of clinical trials have been conducted in the last decade. The first clinical trials comparing intermittent hemodialysis with cellulose membranes and synthetic membranes suggested that the synthetic membranes produced improved outcomes [62]. However, more recent studies have not confirmed these findings (abstracts, Gastaldello et al, *J Am Soc Nephrol* 7:1447, 1996; Albright et al, *J Am Soc Nephrol* 99:197A, 1998; Jorres et al, *J Am Soc Nephrol* 9:212A, 1998). In

one study (abstract, Karsou et al, *J Am Soc Nephrol* 10: 286A, 1999), a meta-analysis of the clinical trials comparing outcomes of patients with ARF dialyzed with cellulose/cellulose-derived membranes or synthetic membranes was performed. In the final analysis, the risk ratio for death was 0.92 (95% confidence interval, 0.76 to 1.13, not significant) comparing cellulose-derived with synthetic membranes. Another recent study addressed the biologic basis of the hypothesis that bioincompatible membranes could increase inflammatory mediators in patients with ARF [48]. In a crossover study, the authors examined the effect of the dialyzer membrane type on cytokine synthesis by PBMCs, superoxide release by neutrophils, and apoptosis of neutrophils. There was no impact of dialyzer biocompatibility on the synthesis of TNFα or IL-10 by PBMCs, superoxide release by neutrophils, or neutrophil apoptosis. This held true regardless of the initial dialyzer assignment. Furthermore, there was no correlation between dialysis adequacy (measured by single pool Kt/V) and post-dialysis cellular responses. Therefore, in this population of very sick, often septic, patients with ARF, bioincompatibility of dialysis membranes seemed to play a minor role in the cellular responses monitored.

In summary, although initial studies suggested that bioincompatible membranes could worsen the outcomes of ARF patients during dialysis, the accumulated data from clinical trials suggest that their role is probably insignificant. Despite the technologic advances in renal replacement therapy techniques, recent data indicate that the dialytic management of ICU patients also can rely on a more conventional option. Indeed, adequate ultrafiltration and good metabolic control were achieved with continuous peritoneal dialysis, even in hypercatabolic patients [abstract; Pecoits-Filho et al, *Perit Dial Int* 20(Suppl 1):S72, 2000]. Peritonitis (10%), hypernatremia, and high serum bicarbonate levels were the main complications, and all were adequately controlled.

Parenteral nutrition

Even while receiving parenteral nutrition, patients with sepsis as the cause of ARF who have a dysfunctioning gastrointestinal tract have a mortality rate of 80% to 90% [63]. These patients are in substantial negative nitrogen balance as a result of their high protein catabolic rates. An important issue is the amount of calories that should be administered to ARF patients. Furthermore, the accuracy of measurements of energy expenditure in such patients is complicated [64]. Therefore, calorimetric methods are often preferred for the assessment of energy expenditure in ARF-MODS patients [65]. Another important issue relates to the composition of the nonprotein component of the diet. Evidence from two decades ago suggests that in sepsis there is a shift in fuel preference to lipids [66]. This information, obtained from experimental and human studies, has led to a progressive increase in the use of intravenous lipid emulsions in such patients.

There is broad agreement that nitrogen administration is necessary and beneficial in critically ill patients. Patients with ARF have urea appearance rates ranging from 7.5 to 18.0 g nitrogen/day [67]. For such highly catabolic patients, however, no optimal level of protein intake has been established that avoids or reduces the need for renal support. Chronic renal failure patients, whose protein catabolism is severalfold lower than that of ARF patients, benefit from parenteral formulas containing essential amino acids as the sole protein source and demonstrate reduced urea production rates and lower dialysis requirements. The biologic rationale of these formulas is that the synthesis of nonessential amino acids retains the nitrogen molecules that would otherwise be excreted. Hence, urea generation is reduced. In the ARF patient, however, the use of essential amino acids has not been effective [68] because of the very high protein catabolic rate.

Nutritional management in ARF also is influenced by the modality of renal support. Many nutrients, including free amino acids, are lost during CRRT and must be replaced by parenteral nutrition. In CVVH, for instance, amino acid loss can be as high as 4 to 7 g/day [69].

Recovery

Most patients who survive an episode of ARF partially or totally recover their renal function. In today's case, the patient was discharged from the hospital with a serum creatinine level of 1.2 mg/dL and a creatinine clearance of 70 mL/min. During the recovery phase, the progressive improvement of glomerular filtration and tubular function gradually increases urinary volume. This complex phenomenon depends on a series of events that allows the anatomic and functional recovery of the renal tissues. The process involves the regeneration of the injured but still viable cells and the removal and replacement of the dead cells that detach from the tubular basement membrane toward the lumen as a result of necrotic and apoptotic mechanisms. Initially, dedifferentiation takes place and marginal cells near the denuded tubular regions acquire an epithelial aspect; then they migrate and multiply in an attempt to completely re-epithelialize the renal tubule [70]. This process involves dynamic changes in the extracellular matrix, the expression and distribution of cytoskeleton, and the membrane proteins. In many aspects, this process recapitulates the cellular mechanisms involved in renal embryogenesis [71].

The recovery phenomenon depends, at least in part, on the participation of growth factors, which, through their autocrine, paracrine, and endocrine actions, can stimulate cellular migration and proliferation in addition to promoting tubulogenesis, thereby re-establishing the integrity of the tubular epithelium [72–74, 75]. Renal

cells exposed to these growth factors or to a harmful event (ischemia or toxins) express a group of immediate early genes in a similar way [76]. Additionally, genes that express HSP (HSP70 and HSP25, for instance) participate in the recovery phase from LPS injury [77, 19].

The exogenous administration of growth factors, especially EGF, IGF-I, and HGF, proved to be beneficial in various experimental ARF protocols by reducing injury, accelerating recovery of renal function, mitigating catabolism, and improving the survival of the treated animals [74, 78]. Recently, we demonstrated in a model of in-vitro hypoxia that HGF, but not EGF or IGF-I, could diminish injury, improve cell viability, and reduce apoptosis of renal tubular cells (abstract, Durão et al, *J Am Soc Nephrol* 9:587A, 1998). Unfortunately, however, the use of IGF in humans with ARF failed to show any beneficial effects in one carefully controlled study [52].

QUESTIONS AND ANSWERS

DR. NICOLAOS E. MADIAS (*Executive Academic Dean, Tufts University School of Medicine, Boston, Massachusetts, USA*): Exciting information has appeared recently on the strong association of the TNF2 polymorphism of the TNFα promoter with susceptibility to septic shock and death due to septic shock [75]. Could you please comment on the potential implications of this and similar polymorphisms of mediators of the sepsis syndrome?

DR. SCHOR: Yes. The multicenter study you mentioned looked for a polymorphism of TNFα promoter and disclosed that the frequency of the TNF2 allele is strongly associated with susceptibility to septic shock and with a high mortality rate [75]. Although this study found an association between the TNF2 allele and mortality, it failed to detect a correlation with plasma TNFα concentration. As I mentioned, despite experimental data indicating that the administration of anti-TNFα antibody or antibody against TNFα receptors protects animals from lethal effects caused by LPS, one rigorous clinical study did not show improvement in survival rates with the use of anti-TNFα antibody [79]. I believe that future clinical trials should target a number of mediators since it is clear that ARF induced by sepsis is a multifactorial disease. Genetic polymorphisms of various cytokines might well be involved in the development or outcome of the sepsis syndrome.

DR. GERHARD MALNIC (*Professor of Physiology and Biophysics, Instituto de Ciências Biológicas, University of São Paulo, São Paulo, Brasil*): Sepsis is one of the leading causes of lactic acidosis, and tissue hypoperfusion and hypoxia are involved in the development of this complication. Of the many steps in the cascade of factors you described, which are important for the development of lactic acidosis? Are there pathogenetic factors besides hypoperfusion?

DR. SCHOR: The physiologic and metabolic manifestations of septic shock include severe hypotension, myocardial dysfunction, organ hypoperfusion, and lactic acidosis. The last of these is mainly a consequence of cellular hypoxia due to alterations in the microcirculation. However, it is possible that LPS per se, or via pro-inflammatory cytokines, such as TNFα or IL-1β, causes mitochondrial dysfunction that aggravates lactic acidosis. In recent years mutations in human mitochondrial DNA have been described that could potentiate lactic acidosis in septicemia [80]. Remember that drugs can affect mitochondrial metabolism, mainly nucleoside analogs (antiretroviral drugs), and their administration in septic patients could exacerbate lactic acidosis.

DR. MIGUEL CENDOROGLO (*Associate Professor of Nephrology, Escola Paulista de Medicine-UNIFESP, São Paulo*): What is your opinion about the importance of the "toll-like receptor 4" in the specificity of innate immunity?

DR. SCHOR: The recent discovery of the toll-like receptor, mainly the Tlr4, has contributed to our understanding of the afferent limb of the innate immune response. As you know, the toxic effects of LPS are indirect; macrophages are the pivotal cells in the signaling mechanisms and resulting biologic responses [11]. Toll receptors, which are plasma membrane receptors on macrophages, essentially function as LPS sensors; they "sense" the presence of LPS, cause activation of NF-κB, and trigger the pro-inflammatory response. It is possible that even modest perturbations of Tlr4 (including altered quantity or post-translational modifications) and/or related molecules profoundly transform the response to LPS. This is the beginning of a new and important field of research of the septic syndrome.

DR. MADIAS: How specific and selective is the down-regulation of the transport molecules that you mentioned in acute renal failure?

DR. SCHOR: Renal ischemia in rats significantly down-regulates Na^+ transporters along the nephron and aquaporins in the collecting duct with no alteration in other membrane proteins; this finding suggests selectivity of ischemia-induced ARF on transport molecules [55]. It is unknown whether other toxic or septic insults induce the same kind of down-regulation. However, as LPS are localized on plasma membranes of the apical microvilli and in the labyrinth of the lateral intercellular spaces, alterations in other transporters also might occur.

DR. LUIS YU (*Associate Professor of Nephrology, University of São Paulo*): Is there any difference in gram-positive and gram-negative sepsis-induced ARF regarding incidence and outcome?

DR. SCHOR: There are no conclusive data on this issue, although increased incidence and mortality of ARF due to gram-positive sepsis compared with gram-negative sepsis have been reported [81].

DR. OMAR DA ROSA SANTOS (*Chairman, Internal Medicine, University of Rio de Janeiro-UNI-Rio, Rio de Janeiro, Brasil*): Oxygen therapy in a hyperbaric chamber has been claimed to be valuable in the treatment of several clinical conditions. What is the clinical indication, if any, for starting hyperbaric oxygen therapy, chiefly in the setting of acute renal failure?

DR. SCHOR: Although there are no specific indications for ARF, some clinical conditions related to ARF could be treated by hyperbaric oxygen therapy, such as necrotizing infection of soft tissues, muscles, and fascia, especially when gaseous gangrene is present. However, early and aggressive surgical exploration is important to remove the necrotic tissue, reduce compartmental pressure, and obtain material for analysis and culture before initiation of hyperbaric treatment. Crush and compartmental syndromes, acute ischemic lesions, and refractory osteomyelitis, which frequently are associated with ARF, also are indications for hyperbaric oxygen treatment. Hyperbaric oxygen therapy for grafted skin, burns, and radiation-induced tissue damage can be useful. Finally, poisoning by cyanide or carbon monoxide, gaseous embolus, and diving decompressive disease are other indications for hyperbaric oxygen therapy.

DR. MADIAS: Given the several potential utilities of renal replacement therapy in the acute renal failure of sepsis, what are your thoughts on the timing of its initiation?

DR. SCHOR: It is clear to me that there is a moderate removal of inflammatory mediators during continuous chronic renal replacement therapy in septic patients, particularly when high volumes of ultrafiltration are achieved. Some clinicians have advocated its use in the early phase of septic ARF, but anti- as well as pro-inflammatory mediators are removed, and the net effect is not known yet. On the other hand, the beneficial effect of high convective clearances also could be due to optimal control of uremic toxicity.

DR. EMMANUEL BURDMANN (*Associate Professor of Nephrology, São José do Roi Preto Medical School, São Paulo*): Would you please comment on the possible role of vancomycin in the development of ARF in this patient?

DR. SCHOR: Vancomycin administration has been associated with ARF due to acute tubular necrosis independently of the concomitant use of other nephrotoxic drugs or hemodynamic alterations. So vancomycin might have played a role in precipitating the ARF in this case. It is interesting to note that experimental and clinical data suggest that vancomycin nephrotoxicity can be reduced with the concomitant use of cilastatin, imipemen, or teicoplamide. At any event, it is important that one adjust the vancomycin dose during CRRT.

DR. MADIAS: You mentioned that a number of immunotherapeutic approaches, for example, anti-LPS mAb, anticytokine strategies, have failed in the treatment of the sepsis syndrome. Do you see a role for extracorporeal adsorptive therapies for removal of microbial mediators of sepsis, such as polymyxin B–linked polymers?

DR. SCHOR: As I mentioned, the problem with these nonspecific therapies is that they can remove both anti- and pro-inflammatory mediators. Polymyxin-B-linked polymers have been used in Japan, and several authors have claimed that they could improve outcome in septic patients. Polymyxin B indeed can remove LPS from the blood and decrease its effect on cytokine generation by mononuclear cells. However, the problem is that these polymers would need to be used in the early phase of sepsis. Also, in spite of the availability of this technique for more than 10 years, no controlled trial has been published. Therefore, I remain skeptical about this technology.

DR. DAVID MACHADO (*Associate Professor of Nephrology, University of São Paulo*): You said that the best renal replacement therapy is the one you know how to do best. But sometimes, the optimal treatment is the one that is available. What is the role of peritoneal dialysis in acute renal failure?

DR. SCHOR: Dr. Noronha's group has shown that using continuous peritoneal dialysis in an intensive care unit can produce metabolic control as well as adequate ultrafiltration, even for hypercatabolic patients [abstract, Pecoits-Filho et al, *Perit Dial Int* 20(Suppl 1):S72, 2000]. As I mentioned earlier, peritonitis (10%), hypernatremia, and high serum bicarbonate were the main complications but were adequately controlled. Therefore, peritoneal dialysis can be utilized as an efficient method for renal replacement, including in acute situations.

DR. MADIAS: Can you discuss the appropriate dose of intermittent hemodialysis or continuous therapies in acute renal failure?

DR. SCHOR: The literature contains little information about this topic. Some recent retrospective studies have suggested a beneficial effect of higher doses of dialysis. However, no prospective study has addressed this question. In 1997, during the ASN meeting in San Antonio, Dr. Schiffl presented interesting results of an interim analysis of an ongoing prospective clinical trial comparing patients assigned to daily hemodialysis with patients assigned to alternate-day dialysis. The analysis showed better survival in patients treated with more frequent dialysis. This study has not yet been published, however. Ronco et al made a similar observation and recommended an increase in the rate of ultrafiltration to improve mortality rates [82].

DR. PAULO BENIGNO BATISTA (*Associate Professor of Nephrology, Escola Baiana de Medicina e Saúde Pública, Salvador, Brasil*): Considering the weight of non-renal variables in the prognosis of death in patients with ARF,

do you think that it is important to develop an ARF-specific prognostic index for these patients?

DR. SCHOR: Yes. The more general prognostic indices do not fit well for ARF. It has been suggested that the ATN-ISS [48] index offers better discrimination than other indices commonly used in ICUs. For general prognosis, the APACHE II score appears to better predict death in patients with ARF at the time of evaluation by the nephrologist than at the time of ICU admission.

DR. BATISTA: What do you think about developing and validating at local, regional, and global levels a specific prognostic index for ARF, independent of the therapeutics, for instance, mechanical ventilation, dialysis, etc.?

DR. SCHOR: Considering the relevance of these indices, we are looking to develop a specific index for our patients in Brasil. Such an indicator would be able to evaluate and compare patients with ARF and provide a prognostic index for patients and groups of patients treated in ICUs with distinct technologic abilities.

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Exhibit 9

Eur J Med Chem (1997) 32, 687–707

Invited review

Cardiac ischaemia: possibilities for future drug therapy

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Summary — This review discusses some of the new avenues of research into cardiac ischaemia being explored by the pharmaceutical industry, which is one of the few areas of cardiovascular research where the industry has maintained a high level of interest. Discussion centres on the possibilities of discovering site-specific coronary vasodilator or antivasoconstrictor agents, drugs capable of restoring normal function to a damaged vascular endothelium or protecting it from ischaemia, agents with beneficial local neurohormonal modulating or metabolic effects and compounds able to modify the remodelling processes of the cardiovascular system triggered by ischaemia. The review gives a non-exhaustive list of the types of structures under investigation in each of these domains.

cardiac ischaemia / future drugs / vascular endothelium / remodelling

Introduction

Given that several classes of drugs provide very effective symptomatic relief of angina pectoris, major advances in therapy will come only with novel compounds able to modify underlying causes of the disease. This will not be easy because the aetiology of CAD is complex and the response of the heart to ischaemia multifaceted. Depending on severity, duration and precipitating factor(s), the heart reacts with acute and chronic compensatory responses to ischaemia. Furthermore, as the disease progresses, these initially homeostatic adaptations contribute to a worsening spiral of events leading to arrhythmia and death [1].

However, recent trials of lipid lowering agents in CAD patients [2], in men with hypercholesterolaemia [3] and in patients with average cholesterol levels following MI [4] have shown significant treatment-related reductions in mortality and in the risk of suffering an ischaemic event. The statins have, therefore, become the first members of a new class of causally effective agents. This review will examine other potential targets which may lead to the anti-ischaemic agents of the future but its scope will not cover the interesting approach of inducing angiogenesis to combat ischaemia [5], or the acute emergency treatment of MI with drugs like thrombolytics, thrombin antagonists, heparin derivatives and antiplatelet agents, although certain glycoprotein IIb/IIIa antagonists do hold promise for preventing the delayed ischaemia associated with restenosis following cardiac surgery (PTCA, CABG and stenting).

Pathophysiology

Cardiac ischaemia occurs when there is an imbalance between demand and supply of oxygen via the coronary arteries. The view that associates stable angina

Abbreviations – A1(2)-agonist(antagonist), A1(2)-adenosine receptor agonist(antagonist); α1(2)-agonist(antagonist), α1(2)-adrenoceptor agonist(antagonist); AMP, adenosine monophosphate; AT-II, angiotensin-II; ACE (I), angiotensin converting enzyme (inhibitor); β-blocker, β-adrenoceptor blocking agent; BP, blood pressure; CA, calcium antagonist; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CHF, congestive heart failure; DHP, 1,4-dihydropyridine; ECG, electrocardiogram; EDRF, endothelium-dependent relaxing factor; ET, endothelin; ELAM-1, endothelial-leucocyte adhesion molecule-1; ETCE(I), endothelin converting enzyme (inhibitor); H_2-receptor, H_2-histamine receptor; HF, heart failure; hOP-1, human osteogenic protein; HR, heart rate; ICAM-1, intercellular adhesion molecule-1; IL-1 (6), interleukin-1 (6); ILGF-1, insulin-like growth factor-1; KATP, ATP sensitive potassium channel; LAD, left anterior descending coronary artery; LDL, low density lipoprotein; 5-LO, 5-lipoxygenase; MI, myocardial infarction; MMP, matrix metalloprotein; MOC, myocardial oxygen consumption; NA, noradrenaline; NHE, Na^+/H^+ exchanger; NO, nitric oxide; VCAM-1, vascular cell adhesion molecule-1; PDE (I), phosphodiesterase (inhibitor); PDGF, platelet derived growth factor; PKC, protein kinase C; PTCA, percutaneous transluminal coronary angioplasty; RAS, renin-angiotensin system; SNS, sympathetic nervous system; TGF, transforming growth factor-β; TNF, tumor necrosis factor-α.

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with fixed atherosclerotic lesions, variant angina with vasospasm and unstable angina with lesions and platelet aggregation/thrombosis, has given way to the concept of mixed ischaemia [6]. Mixed ischaemia may include contributions from all these components some time during the anginal attacks [7]. In the mixed ischaemia hypothesis residual blood flow to the heart is not fixed but fluctuates over the course of the day in response to vasomotor changes and alterations in resistance at the sites of flow-limiting stenoses. Ischaemia may result from exercise when demand exceeds the residual coronary reserve threshold or at rest during episodes of intense vasoconstriction [6].

The stenoses precipitating MI and unstable angina may be relatively minor lesions; (< 50% diameter stenoses [8–11]) and the traditional view that the outcome of CAD is closely linked to stenosis severity is also questioned by results from the recent cholesterol-lowering trials. Drug therapy whilst producing only a small reduction in stenosis severity reduced adverse cardiac events markedly by 40–80% [12]. The most likely mechanisms underlying this beneficial effect of the statins include plaque stabilisation and improvement of endothelial function thus pointing the way to new avenues for future research [12]. Plaque stability is determined by its lipid content and the density of smooth muscle cells and macrophages in the cap. Compounds able to modulate these parameters or having the ability to protect or correct the endothelial dysfunction and impaired release of NO observed in atherosclerosed blood vessels could provide alternative therapeutic strategies for the treatment of CAD.

Current therapy

The treatment of angina pectoris today relies on three drug classes, the β-blockers, the nitrates and the CAs. Whilst all provide symptomatic relief and their efficacy in angina is well established it is only β-blockers lacking intrinsic sympathomimetic activity that have so far been shown to improve prognosis in unstable angina, acute MI and during the immediate post-infarction period. With CAs the effect on prognosis depends on the CA subgroup [13] and on the presence of CHF in the patient population. Monotherapy with nifedipine in patients with unstable angina and acute MI fails to improve prognosis and there may even be a tendency towards adverse effects. Verapamil and diltiazem do improve prognosis in a subset of patients with acute infarction but no signs of CHF [14, 15]. However, in patients with signs of CHF both compounds tend to increase the rate of reinfarction and mortality. With the nitrates a trend towards improved prognosis in acute MI has been shown in some studies but data in patients with CAD is still lacking [16, 17].

One well-accepted concept having an important bearing on future therapeutics has recently been quashed. It has been known for some time that HR is an important independent predictor of adverse cardiovascular events in CAD patients [18–21]. In addition the antianginal effect of the β-blockers has often been attributed to a reduction in HR. The importance placed on HR reduction led recently to the development of a new class of drugs known as specific bradycardic agents or sinus node inhibitors. It was hoped that they would possess the beneficial effects of the β-blockers whilst lacking their untoward negative inotropic effects. The recent surprising results with zatebradine have demonstrated that this is not the case [22].

Zatebradine is a relatively selective blocker of the I_f sarcolemmal current which determines the rate of spontaneous depolarization of the sinoatrial node. Its effect is rate-dependent, reversible and selective since the compound has no effect on cardiac output, vascular resistance or on PR and QRS intervals of the ECG [23–26]. Studies in animals indicated that zatebradine possessed potential antianginal and anti-ischaemic activity [27] which was clearly linked to the bradycardia [28]. However, in a series of placebo-controlled clinical trials in effort angina, zatebradine was found to be no different from placebo and to produce no additional benefit on exercise tolerance in patients receiving nifedipine. This was in spite of a reduction in HR at rest and on exercise equivalent to that observed with a DHP + β-blocker combination, therapy which is known to have additive efficacy [29, 30].

Thus drugs reducing HR alone do not produce therapeutic benefit in patients with chronic stable angina and raise questions on the use of the HR–BP product as an indicator of potential antianginal activity [22]. They also revive questions as to the mechanism of action of β-blockers in angina and the relevance of certain animal models used to detect novel anti-ischaemic drugs.

Alternative therapeutic strategies

Whilst lowering MOC is the mainstay of current therapeutics in chronic stable angina the above clinical findings suggest alternative avenues for future research.

Targeted vasorelaxation

Symptomatic benefit could be obtained with a targeted vasodilator or antivasoconstrictor agent if it prevented the transient increases in vasomotor tone in the affected vessel segment. A generalised, sustained reduction of vascular tone is possible with current

vities. For example, YM-15430-1 (Yamanouchi) is a DHP structure possessing both calcium entry blocking and β-blocking activity [102]. S-2150 (Shonogi), is a combined calcium entry blocker and α1-antagonist which possesses antihypertensive properties [121]. H324/38 (Astra) is another DHP calcium entry blocker but with an ultrashort duration of action that is being developed to treat perioperative hypertension [122]. K201 (Kirin) is a novel benzothiazepine which unlike diltiazem may have an intracellular locus of action since it inhibits annexin binding to F-actin and prevents over-contraction of the myocardium to excessive catecholamine stimulation [123]. SR-33557 (fantofarone, Sanofi) is claimed to act at a novel site in the L-type calcium channel [124, 125] and SL 870495 (Synthélabo), as having little effect upon cardiac conduction [126]. U88999 [127] is a CA with antioxidant activity and GS 386 (Gyeongsang National University) is an isoquinoline derivative claimed to possess effects upon calcium movements intracellularly [128]. Several structures for which specific bradycardic effects are claimed are to be found in the patent literature but the continued development of such agents must be in doubt following the negative results of recent clinical trials with zatebradine (Knoll).

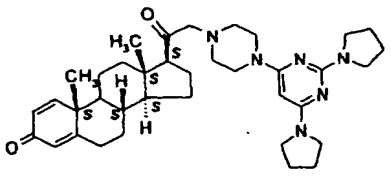
A meta-analysis of secondary prevention trials with nifedipine suggests that high dose, short-acting preparations may be associated with an increased total mortality in patients with CAD [129]. It is now recommended that the use of short-acting agents should be avoided because they induce reflex activation of the SNS. Such reports have elicited much controversy and although the data on individual agents does not allow definitive conclusions they clearly have implications as to whether the pharmaceutical industry will continue to invest valuable resources into the research and development of new CA drugs.

Compounds acting directly on inflammatory or cell adhesion pathways

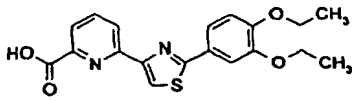
The protective effects of oestradiol against atherosclerosis has been well documented [130] with lipoprotein changes and antioxidant activity being implicated in this effect [131]. However, oestradiol prevents the adhesion of monocytes to endothelial cells [132] and since this is thought to be one of the earliest events in atherosclerosis, it may also provide part of the explanation for the beneficial effects of this hormone. Two other compounds have also recently been claimed to protect the endothelium from ischaemic injury. hOP-1, a member of the TGF β family, has been studied for its anti-ischaemic effects in a rat model of reversible cardiac ischaemia [133]. hOP-1 was found to preserve coronary endothelial function (release of EDRF) following global ischaemia and reperfusion and was observed to significantly inhibit the adherence of neutrophils to the endothelium. Similarly U-74389G (Upjohn) is claimed to improve survival and reverse endothelial dysfunction caused by ischaemia and reperfusion in the splanchnic bed of the rat [134]. Whether similar effects occur at the level of the coronary arteries remains to be determined. The beneficial actions of these agents may be linked to effects upon cell adhesion or inflammatory/oxidant events within the vessel wall. Indeed antioxidant compounds like the thiazole, OPC-6535 (Otsuka) and dithiol-thione compounds from Mitsui-Toatsu (see table VI) are claimed to be useful for the treatment of ischaemic heart disease and a series of membrane bound benzenesulphonamide phospholipase A_2 inhibitors from Eisai have also been reported to reduce the size of occlusion-induced infarcts in the rat when administered before ligation of the coronary artery [135].

From the literature and patents it is evident that many companies have an active interest in the area of adhesion molecule antagonists. Adhesion receptors are expressed following ischaemic injury and reperfusion and are involved in the mediation of leucocyte rolling, adhesion and migration responses through the damaged endothelium [136–138]. Compounds described in the literature (table VI) include a series of dithiocarbamate derivatives (Emory University) claimed to selectively block the expression of VCAM-1 without significant effects upon ICAM-1 and ELAM-1 thus avoiding general immunosuppression. E. Merck also has series of linear and cyclic peptides under investigation which are claimed to act as inhibitors of cell adhesion. Similarly Corvas-Pfizer are studying a neutrophil inhibitory factor which binds to the CDIIb/CD18 integrin cell-surface receptor to prevent neutrophil adhesion and Ligand-Sankyo are working on a series of sialyl Lewis X glycomimetics to prevent P-selectin-induced leucocyte adhesion to the endothelium. LEX032 (Lexin) is a recombinant human protease inhibitor derived from α1-antichymotrypsin in which six amino acids in the centre of the active loop domain have been replaced with those of the α1-protease inhibitor [139]. LEX032 inhibits both neutrophil elastase and cathepsin G, suppresses superoxide generation and attenuates ischaemia and reperfusion-induced injury in the rat, it is claimed through inhibition of neutrophil accumulation in the reperfused zone [140]. Positive results in a similar rat model have been reported with the IL-6 antagonist, KC-12849 (Kalie-Chemie) [141]. However in a canine model of ischaemia and reperfusion the relatively selective 5-LO inhibitor, LY233569 (Lilly) [142], was found to have no significant effect on infarct size or leucocyte recruitment suggesting that leukotrienes do not mediate reperfusion injury in the heart. The

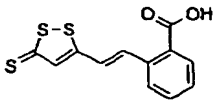
Table VI. Compounds affecting cell adhesion and migration under investigation.



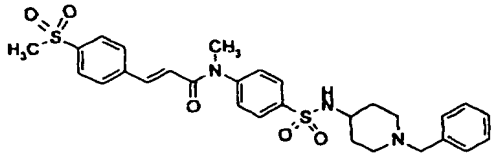
U-74389G (Upjohn)



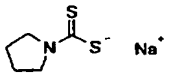
OPC-6535 (Otsuka)



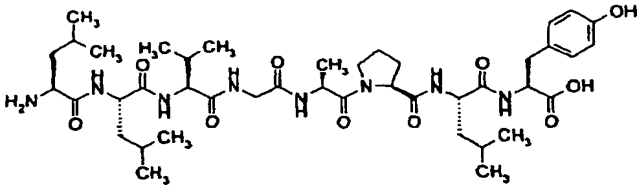
Ditiol-thione compound (MitsuiToatsu)



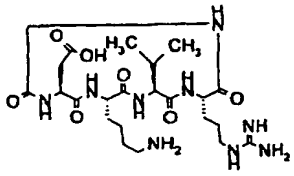
Benzenesulphonamide (Eisai)



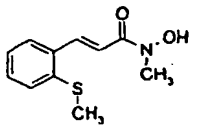
Dithiocarbonate (Emory University)



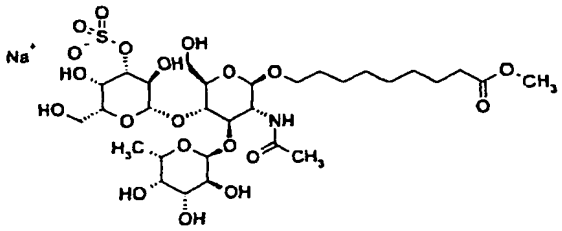
Peptide linear (E.Merck)



Peptide cyclic (E.Merck)



LY-233569 (Lilly)



Sialyl Lewis X glycomimetics (Ligand / Sankyo)

fact that several other 5-LO inhibitors [143–146] have all been reported to salvage ischaemic myocardium and to limit leucocyte accumulation has been attributed to a lack of enzyme selectivity of these agents (eg, inhibition of cyclooxygenase) or additional pharmacological effects (eg, antioxidant activity) [147, 148].

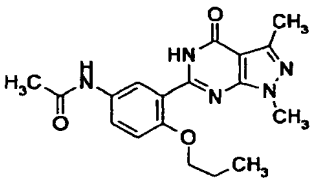
Finally several companies are exploring the anti-ischaemic potential of selective inhibitors of the PDE isoenzymes, especially inhibitors of PDE III and V, probably because of their potential selectivity for vascular smooth muscle and platelets [149]. Glaxo Wellcome is studying a series of pyrazolopyrimidines (table VII) and both Pfizer and SmithKline Beecham are working on calmodulin-insensitive, cyclic GMP-dependent PDE III inhibitors with similar pharmacological profiles (antiaggregant, vasorelaxant activities). In this respect the relatively selective PDE III inhibitor, BMY21190 (Bristol Meyers Squibb) has been reported to reduce infarct size in the canine myocardium following 90 minutes of ischaemia and reperfusion via inhibition of neutrophil infiltration [150]. Following the demise of PDE-I inhibitors in CHF it remains to be seen if any acute improvements in physiological function observed with these drugs will translate into real long-term clinical benefits.

Local neurohormonal or metabolic effects

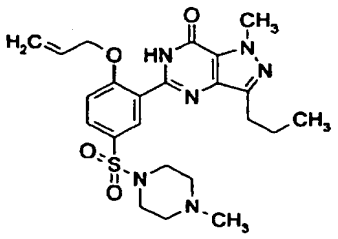
Ischaemia excites certain cardiac afferents which then provoke centrally mediated increases in SNS activity in the periphery. Patients undergoing CABG operations are at risk of myocardial cell injury and several pharmacological approaches have been used in attempts to reduce the incidence of peri- and post-operative myocardial ischaemia. In such situations the α2-agonists clonidine [151], dexmedetomidine [152] and more recently mivazerol [153] have been reported to have beneficial effects (table VIII). Mivazerol, is a relatively selective α2-agonist [154] with little effect on BP, which might be advantageous for this indication [155]. Mivazerol is effective in reducing increases in T-wave amplitude provoked by brief periods of coronary occlusion in anaesthetised rats and dogs [156, 157] and suppresses the tachycardia following emergence from halothane anaesthesia in a rat model [158]. The effect of mivazerol is probably due to a reduction in peripheral SNS activity mediated by α2-receptor stimulation since its effects are abolished by pretreatment with rauwolscine. Studies into the site of action of mivazerol are still ongoing but there is evidence that spinal and ganglionic sites may be involved. Mivazerol is in the final phases of clinical development (Phase III) as a protective agent for use in CAD disease patients undergoing non-cardiac surgery [153].

The therapeutic place of β-blocking drugs possessing additional vasodilator properties like YM-15430-1 is still unclear [120, 159]. Such a combination of activities may be beneficial from a haemodynamic standpoint and with carvedilol [160], for example, this has translated into significant clinical benefits since it

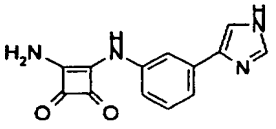
Table VII. Selected phosphodiesterase inhibitors under study.



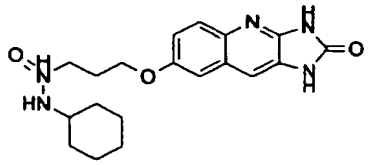
Pyrazolopyrimidine (Glaxo Wellcome)



Pyrazolopyrimidine (Pfizer)



Pyridylamino-cyclobutene dione (Smith Kline Beecham)



BMY 21190
(Bristol Meyers Squibb)

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Inhibition of leukotriene release in anaphylactic guinea-pig hearts by a 5-lipoxygenase inhibitor, CGS 8515

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1 Ovalbumen (100 μg)-induced coronary vasoconstriction and decrease in cardiac developed tension were studied in isolated perfused hearts from sensitized guinea-pigs. Leukotriene-like material released in the cardiac effluent was assayed against synthetic leukotriene C_4 (LTC_4).

2 LTC_4 was released in a time-dependent fashion, and release was enhanced when hearts were challenged in the presence of indomethacin (2.8 μM). The release was maximal at 2–3 min and detectable for as long as 10 min following ovalbumen challenge. Immunoreactive (ir) thromboxane-B_2 (TxE_2) was also detected in cardiac effluent which had been partially purified using C_{18} Sep-Paks.

3 CGS 8515 (0.03–1.0 μM), an inhibitor of 5-lipoxygenase, dose-dependently inhibited ovalbumen-induced coronary vasoconstriction and leukotriene-C_4 release. CGS 8515 inhibited ovalbumen-induced decreases in cardiac developed tension at 0.3 and 1.0 μM, but did not antagonize coronary vasoconstriction induced by synthetic LTC_4.

4 The release of cyclo-oxygenase products following ovalbumen challenge was not inhibited by CGS 8515, but was markedly inhibited by indomethacin (2.8 μM) pretreatment.

5 We conclude that leukotrienes have a major role in guinea-pig cardiac anaphylaxis, and that CGS 8515 has a cardio-protective action. The results obtained in these experiments *in vitro* show that CGS 8515 is a potent and selective 5-lipoxygenase inhibitor.

Introduction

Hypotensive shock and cardiac failure are clinical manifestations of systemic anaphylaxis (Austen, 1974). The anaphylactic reaction of isolated sensitized guinea-pig hearts shows resemblance to human anaphylaxis. It is characterized by the occurrence of typical symptoms such as coronary vasoconstriction, cardiac failure and arrhythmias (Hahn & Bernauer, 1970; Capurro & Levi, 1975; Liebig *et al.*, 1975; Levi & Burke, 1981). Slow reacting substance of anaphylaxis, now known to consist of leukotrienes (LTs) C_4, D_4 and E_4, has been detected in effluent of anaphylactic guinea-pig hearts and is believed to be responsible for the changes in cardiac function observed (Brocklehurst, 1960; Chakravarty, 1960). Indeed, partially purified SRS-A obtained immunologically from guinea-pig or synthetic LTC_4, LTD_4 and LTE_4 cause significant reductions in cardiac developed tension and coronary flow (Letts & Piper, 1982).

As 5-lipoxygenase products are among the important mediators released during cardiac anaphylaxis, one possible way of reducing the symptoms of cardiac dysfunction and hence the eventual cardiac failure secondary to cardiac anaphylaxis, may be by inhibiting the lipoxygenase enzymes. It will therefore be of interest to investigate the therapeutic effect of selective inhibitors of 5-lipoxygenase in cardiac anaphylaxis. There are, however, relatively few reports of specific 5-lipoxygenase inhibitors with activity both *in vivo* and *in vitro*. Evidence of inhibition of 5-lipoxygenase both *in vivo* and *in vitro* has been obtained with BW755c (Hammarstrom, 1977) but this compound is not specific for 5-lipoxygenase and also inhibits cyclo-oxygenase.

Most of the investigations carried out with this drug have been on the inhibition of various inflammatory responses. We have investigated the pharmacological activities of CGS 8515, a potent lipoxygenase inhibitor, on the release of LTC_4-like material from guinea-pig hearts during cardiac anaphylaxis and on the associated increase in coronary perfusion pressure and decrease in cardiac developed tension.

Methods

Sensitization

Male weanling guinea-pigs of Dunkin-Hartley strain (200–250 g) were sensitized with doses of crude egg albumen (Sigma grade II) 100 mg subcutaneously and 100 mg intraperitoneally.

Heart perfusion

Three to four weeks after sensitization, each animal was killed by cervical dislocation, the heart removed and placed in cold Tyrode solution. The aorta was cannulated retrogradely, the heart rapidly transferred to the modified Langendorf perfusion apparatus where it was immediately perfused with oxygenated Tyrode solution at a constant flow of 10 ml min^{-1}. Coronary perfusion pressure and cardiac developed tension were recorded continuously on a Watanabe linearcorder mark VII WR3101. After an equilibration period of 30 min, the heart was challenged with a bolus injection of ovalbumen given into the aorta. Cardiac effluent was collected on ice at 1 min intervals for measurement of the release of LTC_4, and thromboxane B_2 (TxB_2).

Drug infusions

Indomethacin, BW 755c and CGS 8515 were each added into the Tyrode solution to give the final concentrations shown below. Indomethacin dissolved in sodium carbonate was added to Tyrode solution to give a final concentration of 1 $\mu g\,ml^{-1}$ (2.8 μM). This concentration has been shown previously to inhibit almost completely the release of cyclo-oxygenase products during cardiac anaphylaxis (Anhut *et al.*, 1977). CGS 8515 was dissolved in N-N-dimethylacetamide at a concentration of 10 mM, which was then diluted in Tyrode solution to give concentrations of 0.03, 0.1, 0.3 and 1.0 μM. At the concentrations used, the vehicle N-N-dimethylacetamide had no measurable effect on the hearts. BW755c was dissolved in distilled water and the desired concentration was obtained by dilution in Tyrode solution as above.

Bioassay of leukotriene C_4

Leukotriene-like material in the cardiac effluent was assayed against synthetic LTC_4 on strips of longitudinal smooth muscle of the guinea-pig ileum (Rang, 1964), superfused at 5 ml min^{-1} with oxygenated Tyrode solution warmed to 37°C, containing the following combination of antagonists: mepyramine (0.1 $\mu g\,ml^{-1}$), hyoscine (0.1 $\mu g\,ml^{-1}$), methysergide (0.2 $\mu g\,ml^{-1}$), phenoxybenzamine (0.1 $\mu g\,ml^{-1}$) and propranolol (2 $\mu g\,ml^{-1}$). Changes in length of tissues were measured by means of auxotonic levers (Paton) connected to smooth muscle transducers (Harvard) and displayed on a pen recorder. Limits of LTC_4 detection were 1.0–3.0 pmol.

Radioimmunoassay

Immunoreactive TxB_2 in the heart effluent was determined by radioimmunoassays as described by Jose *et al.* (1976). Immunoreactive TxB_2 was measured in selected samples 1–10 min before and after challenge. Effluent from the heart was extracted twice with methanol, evaporated to dryness under vacuum and kept frozen until required. The extract was dissolved in standard buffer solution (pH 7) for radioimmunoassay. The assays were carried out using an antiserum to TxB_2. The limit of detection of TxB_2 was 0.01 pmol.

Materials

The following drugs were used: methyl-2-(3,4-dihydro-3,4-dioxo-1-napthalenyl)amino benzoate (CGS 8515, Ciba Geigy), indomethacin (Sigma), 3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2 pyrazoline (BW755c, Wellcome), 7-[3-(4-acetyl-3-hydroxy-2-propyl-phenoxy)-2-hydroxyproboxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylic acid (FPL 55712, Fisons Ltd.), platelet activating factor (Paf) (Bachem), LTC_4 (Miles Laboratories), hyoscine hydrobromide (BDH), mepyramine maleate (May & Baker), methysergide maleate (Sandoz), phenoxybenzamine hydrochloride (ICI), propranolol hydrochloride (ICI), TxB_2, antiserum to TxB_2, egg albumen grade II and III (Sigma).

Results

Effects of vehicle and CGS 8515 on hearts

Infusion of vehicle (0.01% N-N-dimethylacetamide) for CGS 8515 into the heart was without effect and did not significantly alter the resting level of the coronary perfusion pressure (CPP) and cardiac developed tension.

CGS 8515 at concentrations of 0.03, 0.1, and 0.3 μM exerted no significant effect on CPP in the perfused guinea-pig heart. At 1.0 μM CGS 8515 caused a marked increase in resting CPP ($P < 0.05$, $n = 4$) compared with the control hearts. In addition, it was also observed that CGS 8515 exerted a direct effect on the developed tension. At low doses it enhanced whereas at high doses it reduced the developed tension.

Effects of CGS 8515 pretreatment in anaphylactic hearts

Bolus injection of ovalbumen (100 μg) into the fluid perfusing the isolated sensitized guinea-pig hearts

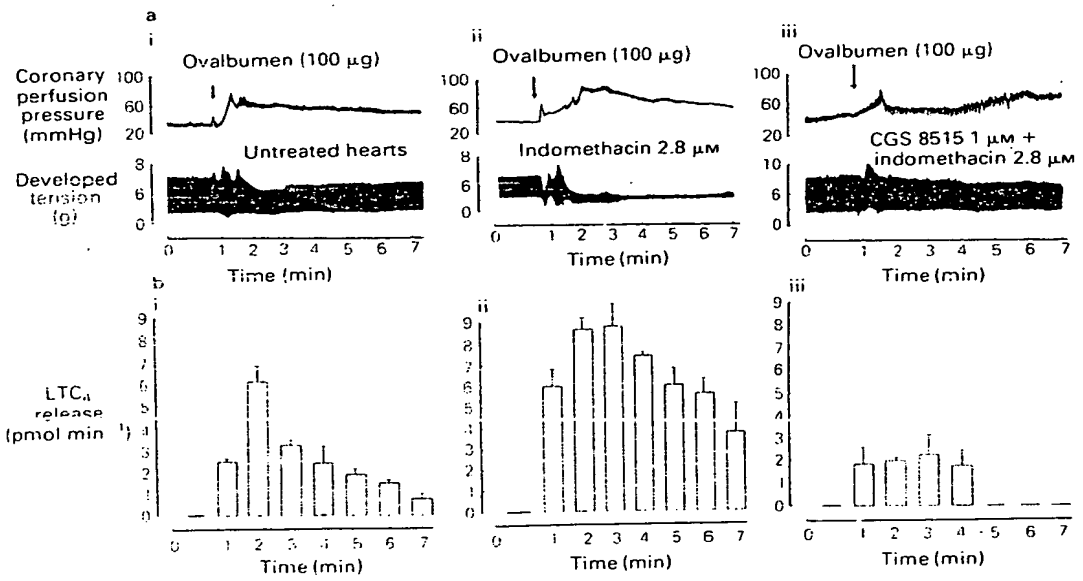

Figure 1 Effects of ovalbumen challenge (100 μg) on coronary perfusion pressure and cardiac developed tension. (a) Representative tracings from four separate experiments in untreated hearts (i), indomethacin treated hearts (ii), and hearts treated with CGS 8515 in the presence of indomethacin (iii). (b) Mean data for the experiments shown in a(i), a(ii) and a(iii).

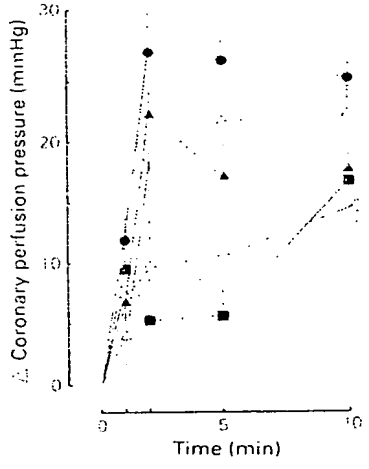

Figure 2 Indomethacin-induced potentiation of the anaphylactic increase in coronary perfusion pressure and its attenuation by different concentrations of CGS 8515 administered in the presence of indomethacin: indomethacin 2.8 μM (●); indomethacin plus CGS 8515: 0.03 μM (△), 0.1 μM (▲), 0.3 μM (◇) and 1.0 μM (■). Each point represents the mean of at least 4 experiments and vertical lines show s.e.mean ($P < 0.05$).

elicited an immediate increase in CPP and a decrease in cardiac developed tension (Figure 1a). The most severe changes occurred 1–2 min after antigen challenge. The peak increase in CPP occurred within 2 to 3 min and remained elevated above control levels for longer than 10 min. The decrease in cardiac developed tension also persisted over the 10 min observation period. Ovalbumen-induced increase in CPP and decrease in cardiac developed tension were altered by indomethacin and BW755c (40 μM) pretreatment. Indomethacin delayed the onset of the early phase of coronary vasoconstriction which occurred at 1 min after antigen challenge (Figure 1), and potentiated the delayed elevation of CPP ($P < 0.0025$, $n = 8$). It was also noted that the incidence of post-challenge atrial fibrillation occurred more frequently in the indomethacin-treated hearts (1 in 2 hearts) than in untreated hearts. Both CGS 8515 and BW755c when infused into indomethacin-treated hearts markedly inhibited both ovalbumen-induced increase in CPP and decrease in cardiac developed tension. The inhibition in the increase in CPP (Figures 2 and 3) was most marked in the early phase (0.5 min) after ovalbumen challenge. A dose-dependent inhibition was observed with CGS 8515 at concentrations between 0.03 and 1.0 μM. At these concentrations CGS 8515 also produced a dose-dependent inhibition of the cardiac developed tension ($P < 0.05$, $n = 4$, and

$P < 0.025$, $n = 4$). CGS 8515 at 0.03 and 0.1 μM did not significantly alter the decrease in cardiac developed tension. The inhibitory effects of CGS 8515 were markedly more potent than those obtained with BW755c (40 μM).

Effect of CGS 8515 on antigen-induced release of 5-lipoxygenase and cyclo-oxygenase products

Figure 1 shows that cysteinyl-containing leukotrienes assayed against LTC_4 were released during cardiac anaphylaxis in a time-dependent manner. Maximum release occurred within 2–3 min but output of leukotriene-like material could be detected for at least 8 min. Treatment of hearts with indomethacin (2.8 μM) (Figure 1, iia,b) substantially increased the release of leukotriene-like material. Perfusion of CGS 8515 either alone or in the presence of indomethacin (Figure 1, iii a,b, and Figure 2) produced a concentration-dependent inhibition of release of leukotriene-like substances. At concentrations of 0.1, 0.3 and 1.0 μM CGS 8515, the level of inhibition was 44%, 66%, and 78%, respectively. On the other hand the inhibition produced by BW755c was 67% (Figure 3). In these experiments the amount of leukotriene-like material released was significantly lower than in the indomethacin-treated hearts (Figures 1 and 5).

While the concentrations of TxB_2 in the hearts collected before challenge were close to or below the detection limit of radioimmunoassay, greatly increased amounts of this mediator were released from the hearts after ovalbumen challenge (13.7 $\pm$ 0.08 pmol min^{-1}). In the presence of indomethacin release of TxB_2 was markedly reduced (Figure 4), while, as shown above (Figure 1, ii a,b) release of cysteinyl-containing leukotrienes, assayed against synthetic LTC_4, was significantly enhanced. CGS 8515 (0.03–1.0 μM) did not inhibit antigen-induced release of the cyclo-oxygenase product TxB_2 (Figure 4), suggesting that CGS 8515 is a selective 5-lipoxygenase inhibitor.

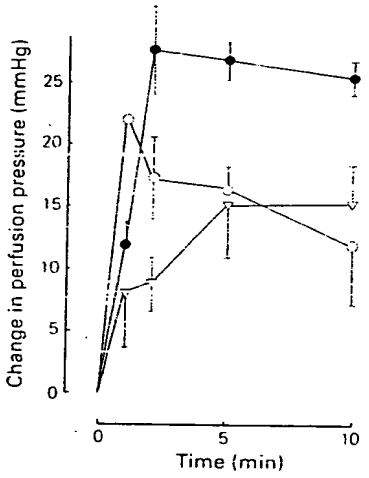

Figure 3 Enhancement of ovalbumen-induced increases in coronary perfusion pressure by indomethacin, 2.8 μM, (●) and its attenuation by BW755c, 40 μM, (▽); untreated heart (○). ($n = 4$–6, $P < 0.05$).

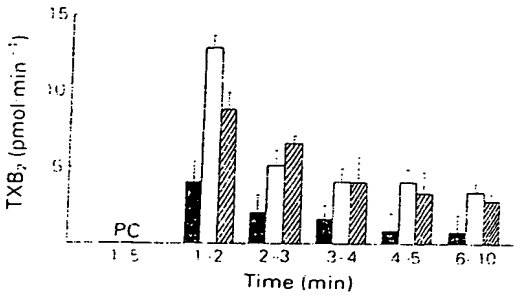

Figure 4 Generation of antigen-induced thromboxane B_2 (TxB_2) in untreated hearts (open columns), and hearts treated with indomethacin (2.8 μM) (solid columns) and with CGS 8515 (1 μM) (hatched columns). PC = prechallenge. Cardiac effluent was collected for periods shown on axis. ($n = 4$, $P < 0.05$).

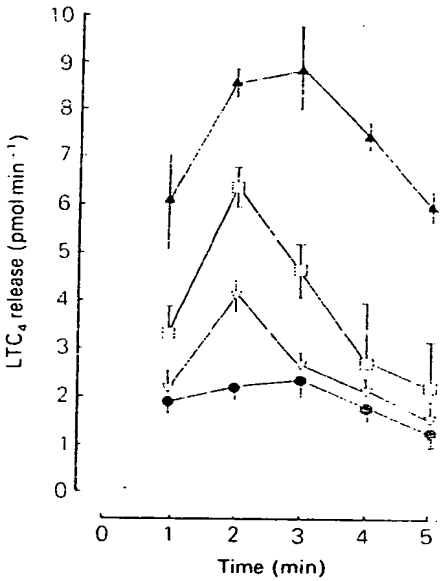

Figure 5 The effects of BW755c (40 μM) and CGS 8515 (1 μM) on the indomethacin-induced exaggeration in the anaphylactic release of leukotriene C_4 (LTC_4)-like material: indomethacin 2.8 μM (▲); untreated hearts (□); BW755c plus indomethacin (▽); CGS 8515 plus indomethacin (●). Cardiac effluent was sampled at 1 min intervals over 5 min.

The effect of CGS 8515 on LTC_4-induced coronary vasoconstriction

Infusions of LTC_4 (40–100 pmol) produced dose-dependent decreases in coronary flow and cardiac developed tension in guinea-pig isolated hearts. CGS 8515 (0.03–1.0 pmol) did not significantly attenuate these responses suggesting that this 5-lipoxygenase inhibitor has no antagonistic activity on leukotriene-receptors.

Discussion

Various mediators have been implicated in cardiac anaphylaxis. Technological advances during the past decade have revealed that lipoxygenation of arachidonic acid (AA) gives rise to a number of products with diverse biological activities and in particular, the discovery of leukotrienes as active constituents of SRS-A released immunologically from guinea-pig hearts (Aehringhaus *et al.*, 1984) has enabled us to elucidate further the mediators involved in cardiac anaphylaxis.

Allergens have been shown to be potent stimuli for the release of vasoactive arachidonic acid metabolites in hypersensitized subjects (Chakravarty, 1960). In the present experiments, guinea-pig hearts isolated from sensitized animals undergo a profound reaction to a bolus injection of ovalbumen resulting in sustained reduction in coronary flow coupled with dysrhythmias and marked impairment of cardiac function (Liebig *et al.*, 1975; Levi *et al.*, 1976). Irrespective of the presence or absence of indomethacin, the maximum release of leukotrienes occurred at 2–3 min after ovalbumen challenge during which time it was noted that the reduction in coronary flow and developed tension of the perfused hearts were maximal. It was of interest that the quantity and duration of leukotriene release significantly increased in the presence of indomethacin. These results suggest that leukotrienes may be partly responsible for cardiac dysfunction seen during anaphylactic shock.

In the present experiments the potency and activity of CGS 8515 against 5-lipoxygenase is manifested by its ability to inhibit ovalbumen-induced release of leukotrienes and the attenuation of the associated increase in CPP and reduction in cardiac developed tension.

CGS 8515 dose-dependently inhibited release of leukotriene-like material and concomitantly inhibited cardiac reactions to ovalbumen. In line with the above observations the effects of CGS 8515 were most marked at 1–5 min following ovalbumen challenge.

The potentiation of the anaphylactic release of SRS-A by indomethacin has been demonstrated in other laboratories (Liebig *et al.*, 1975); however, the significance of this effect on cardiac function is not known. The present experiments demonstrate that a cyclo-oxygenase inhibitor could not only alter mediator release during cardiac anaphylaxis but could also modify cardiac responses in response to antigen challenge. The delay of inhibition of the early phase of antigen-induced vasoconstriction by indomethacin suggests that cyclo-oxyegenase products are significantly involved in cardiac anaphylaxis. In addition, it is interesting to observe that pretreatment with indomethacin resulted in a higher than normal incidence of post-challenge atrial fibrillation. This is in apparent agreement with the previous observation that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) completely suppressed the antigen-induced arrhythmias while PGE_2 and PGI_2 decreased the incidence of arrhythmias in guinea-pig anaphylactic hearts (Aehringhaus *et al.*, 1984). Thus, the enhancement in the anaphylactic release of LTC_4 when sensitized hearts were challenged in the presence of indomethacin seen in the present experiments suggests two possibilities: firstly, cyclo-oxygenase products may exert a direct negative feedback on the anaphylactic release of leukotrienes and secondly, blocking the cyclo-oxygenase pathway resulted in a diversion of arachidonic acid metabolism away from cyclo-oxygenase towards the synthesis of lipoxygenase products. Based on experimental data from other laboratories, the latter possibility would explain our observation since it has been demonstrated that a range of cyclo-oxygenase metabolites has no effect on the anaphylactic release of LTC_4 in indomethacin-treated tissue; this included both vasodilator (PGE_2, PGI_2) and vasoconstrictor substances ($PGF_{2\alpha}$, PGD_2, and 11-9-epoxy-methano-PGH_2, TxA_2-mimetic) (Aehringhaus *et al.*, 1984).

The duration of increase in CPP following a bolus injection of exogenous LTC_4 (30 pmol) was 4 min. On the other hand, the observed increase in coronary perfusion pressure following ovalbumen challenge remained elevated for more than 10 min. This is consistent with the duration of release of LTC_4-like material seen in the present experiments, in which the anaphylactic release of LTC_4-like material was detectable up to 7–10 min. However, the reason for the disparity in the duration of release of LTC_4-like material and the protracted nature of the elevation of CPP following ovalbumen challenge is not known. It remains a possibility that the sustained elevation in perfusion pressure seen above is attributable to the action of vasoactive mediators other than leukotrienes. For instance, platelet activating factor (Paf), a putative mediator of inflammation has recently been implicated in cardiac anaphylaxis (Levi *et al.*, 1984). The protracted nature of Paf-induced increases in CPP and decreases in

cardiac developed tension, in addition to the demonstration that Paf-acether triggered the release of leukotrienes into the cardiac effluent (Piper & Stewart, 1986) makes Paf the most likely mediator responsible for the persistent coronary vasoconstriction seen during cardiac anaphylaxis. Furthermore, CGS 8515 at doses that produced significant attenuation of the increase in CPP only exhibited its marked inhibitory effects between 2–5 min following ovalbumen challenge.

The availability of a new lipoxygenase inhibitor allows participation of lipoxygenase-derived products in cardiac anaphylaxis and other pathophysiological conditions to be assessed. CGS 8515 has been reported to inhibit 5-HETE and LTB_4 synthesis in guinea-pig polymorphonuclear leukocytes (IC_{50} = 0.1 μM) *in vitro* (Ku,*et al.*, 1988). In rats, CGS 8515 at oral doses of 5 mg kg^{-1} inhibits the production of LTB_4 from whole blood *in vivo* by about 50% (Ku *et al.*, 1988). CGS 8515 has also been reported to inhibit leukocyte accumulation in carrageenin-impregnated rats (Ku *et al.*, 1988). The dose-dependent inhibition of the LTC_4-like material released by CGS 8515 at very low doses in the present experiments suggest that CGS 8515 is indeed a potent 5-lipoxygenase inhibitor. In addition, the amounts of BW755c and CGS 8515 required to inhibit approximately 66% of the LTC_4-like material released were 40 μM and 0.3 μM, respectively, showing that the potency of CGS 8515 is more than ten times greater than BW755c.

The absence of significant attenuation of the actions of infused synthetic LTC_4 in the perfused hearts by CGS 8515 suggests that the effects of CGS 8515 seen during cardiac anaphylaxis are not through antagonism of leukotriene receptors but through selective inhibition of the formation of 5-lipoxygenase products. Unlike BW755c, CGS 8515 is a selective 5-lipoxygenase inhibitor and our data show that it has a negligible effect on the release of ir-TxB_2. This supports the findings in other laboratories that CGS 8515 did not inhibit PGE_2 and 15-HETE formation in cell-free systems (Ku *et al.*, 1988).

At present, there are few selective 5-lipoxygenase inhibitors with activity both *in vivo* and *in vitro*. Various acetylenic analogues of arachidonic acid inhibit different lipoxygenases *in vitro*, but this type of compound is not active *in vivo* (Higgs & Mudgridge, 1982). Several compounds inhibit various lipoxygenases *in vitro*. These include nordihydroguiaretic acid (NDGA), benoxaprofen, baicalein, esculetin, some flavonoids and caffeic acid (Hamberg, 1976; Walker & Dawson, 1980; Sekiya & Okuda, 1982). There are however, relatively few reports of selective and potent 5-lipoxygenase inhibitors with activity *in vivo*. Benoxaprofen for instance, while inhibiting 5-lipoxygenase in isolated leukocytes (Harvey *et al.*, 1983) had no effect on LTB_4 generation in acute experimental inflammation (Salmon *et al.*, 1984). Thus, CGS 8515 showing the above properties and having activity both *in vivo* and *in vitro* may be a useful tool with which to elucidate further the role of 5-lipoxygenase products in pathological and physiological processes. Moreover, CGS 8515 has been shown to inhibit selectively the formation of pro-inflammatory agents, LTB_4 and 5-HETE without affecting the release of 15-HETE (Ku *et al.*, 1988). Thus, the results of these experiments, again emphasize the potential therapeutic significance of CGS 8515 in various allergic, inflammatory and pathological conditions.

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Production of leukotrienes in a model of focal cerebral ischaemia in the rat

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1 The aim of this work was to evaluate the role of leukotrienes in brain damage *in vivo* in a model of focal cerebral ischaemia in the rat, obtained by permanent occlusion of middle cerebral artery.

2 A significant ($P<0.01$) elevation of LTC_4, LTD_4 and LTE_4 (cysteinyl-leukotrienes) levels occurred 4 h after ischaemia induction in the ipsilateral cortices of ischaemic compared to sham-operated animals (3998 ± 475 and 897 ± 170 fmol g^{-1} tissue, respectively, $P<0.01$).

3 The NMDA receptor antagonist MK-801 and the adenosine A_{2A} receptor antagonist SCH 58261 were administered *in vivo* at doses known to reduce infarct size and compared with the leukotriene biosynthesis inhibitor MK-886.

4 MK-886 (0.3 and 2 mg kg^{-1} i.v.) and MK-801 (3 mg kg^{-1} i.p.) decreased cysteinyl-leukotriene levels (-78%, $P<0.05$; -100%, $P<0.01$; -92%, $P<0.01$, respectively) 4 h after permanent occlusion of the middle cerebral artery, whereas SCH 58261 (0.01 mg kg^{-1} i.v.) had no significant effects.

5 MK-886 (2 mg kg^{-1} i.v.) was also able to significantly reduce the cortical infarct size by 30% ($P<0.05$).

6 We conclude that cysteinyl-leukotriene formation is associated with NMDA receptor activation, and that it represents a neurotoxic event, the inhibition of which is able to reduce brain infarct area in a focal ischaemic event.

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Keywords: Leukotrienes; cysteinyl-leukotrienes; cerebral infarct; focal cerebral ischaemia; MK-886; MK-801; SCH 58261

Abbreviations: 5-LOX, 5-lipoxygenase; $cPLA_2$, cytosolic phospholipase A_2; cysteinyl-LT, cysteinyl-leukotriene; DMSO, dimethyl sulphoxide; FLAP, five lipoxygenase activating protein; pMCAo, permanent occlusion of the middle cerebral artery

Introduction

Cysteine-containing LTs (cysteinyl-LTs), namely LTC_4, LTD_4, LTE_4 are metabolites of arachidonic acid formed through the 5-lipoxygenase (5-LOX) pathway (Samuelsson, 1983). Cysteinyl-LTs are known mainly for their potent action as constrictors of smooth muscle (Feuerstein, 1985). However, their profile of action is much broader; they possess pro-inflammatory characteristics (Hay *et al.*, 1995) and, in particular, they increase postcapillary venule tone and permeability, thus causing oedema (Dahlen *et al.*, 1981).

Leukotrienes are synthesized in different areas of the central nervous system both *in vitro* and *in vivo* (Dembinska-Kiec *et al.*, 1984; Lindgren *et al.*, 1984). The concentration of free arachidonic acid, which is usually very low in the brain, increases greatly following various stimuli, including ischaemia (Bosisio *et al.*, 1976). Increased LT formation from brain homogenates has been demonstrated in different species where global ischaemia had been induced by bilateral occlusion of common carotid artery, especially after reperfusion (Dempsey *et al.*, 1986a,b; Mabe *et al.*, 1990; Minamisawa *et al.*, 1988; Moskowitz *et al.*, 1984). In addition, elevated cysteinyl-LT levels have been found in cerebrospinal fluid of patients within 72 h from acute cerebral ischaemia (Aktan *et al.*, 1991).

Furthermore, it has been recently suggested that eicosanoids might be associated with neuronal injury after hypoxia or trauma. Indeed, mice deficient in cytosolic phospholipase A_2 ($cPLA_2$), one of the enzymes responsible for arachidonic acid release, had smaller cerebral infarct volume following transient ischaemia (Bonventre *et al.*, 1997) and the excitotoxic aminoacid glutamate enhances PLA_2 activity (Bonventre, 1997). However, there is only indirect evidence, based on the use of the dual cycloxygenase/lipoxygenase inhibitor BW755C, that lipoxygenase products might be involved in neurotoxicity (Baran *et al.*, 1994; Chen *et al.*, 1995).

For a better understanding of the involvement of leukotrienes in ischaemic damage and neurotoxicity, we have chosen a rat model of brain ischaemia induced by the permanent occlusion of the middle cerebral artery (pMCAo). This experimental model is relevant to human stroke, as this pathology is most frequently caused by thrombotic occlusion of the same vessel. The histopathological consequence is a necrotic area involving both neurons and glial cells (the infarct *core*) which develops around the site of occlusion of the MCA. This area is surrounded by the so called *penumbra*, where a secondary damage develops.

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In this model, we examined the effects of two neuroprotectant agents, MK-801 and SCH 58261, on the levels of immunoreactive cysteinyl-LTs (i-cysteinyl-LTs) *in vivo*. MK-801 is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist (Wong *et al.*, 1986) and SCH 58261 is a potent and selective adenosine A_{2A} receptor antagonist (Zocchi *et al.*, 1996). Both these compounds have been shown to have neuroprotective properties in models of focal cerebral ischaemia (Monopoli *et al.*, 1998; Park *et al.*, 1988). Finally, we wanted to see if the biosynthesis of i-cysteinyl-LT triggered by cerebral ischaemia is able *per se* to induce cell death. With this aim we designed experiments with MK-886 (Gillard *et al.*, 1989), a potent and selective inhibitor of the 5-lipoxygenase (IC_{50}: 2.5 nM in isolated human leucocytes), in cerebral ischaemia.

The present study provides the first evidence of neuroprotection obtained by post-ischaemic reduction of leukotriene levels and of the deleterious effects of these lipid mediators, in a model of focal cerebral ischaemia.

Methods

Materials

^{3}H-LTC_4 (specific activity 110.5 Ci mmol^{-1}) were from NEN Life Science Products, Boston, MA, U.S.A. and Sep-Pak C18 solid phase cartridges from Waters Associates, Milford, MA, U.S.A.; n-hexane, EtOAc, MeOH and EtOH for liquid chromatography were purchased from Merck, Darmstadt, Germany; Ultrapure H_2O (MilliQ) was from Millipore Co., Bedford, MA, U.S.A.; A23187 was from Sigma, St. Louis, MO, U.S.A. Reagents for enzyme immunoassay (EIA) were obtained from Cayman Chemical Co., Ann Arbor, MI, U.S.A. except for cysteinyl-LT antibody which was from Perspective Biosystems Inc., Framingham, MA, U.S.A. MK-801 was from R.B.I., Natick, MA, U.S.A.; SCH 58261 was synthesized at the Schering-Plough Research Institute and MK-886 was a kind gift from Dr A. Ford-Hutchinson (Merck-Frosst Canada Inc., Pointe-Claire, Dorval, Quebec, Canada). Ultima Gold was from Packard Instruments Co., Meriden, CT, U.S.A.

Animal housing and surgery

Experiments were conducted in male Sprague-Dawley rats (Charles River, Calco, Como, Italy), weighing 250–275 g. Procedures involving animals and their care were conducted in conformity with the institutional guidelines, in compliance with the European Economic Community Council Directive 86/609 (OJ L 358, 1, December 12, 1987). The animals were caged for at least 3 days before surgery, with free access to food (until 12 h before surgery) and water, and maintained on a 12 light/12 dark schedule (lights on at 0700 h).

Focal cerebral ischaemia was induced by permanent, unilateral occlusion of the left middle cerebral artery (pMCAo) in rats anaesthetized with chloral hydrate (400 mg kg^{-1} i.p.). The pMCAo was performed according to methods described elsewhere with minor changes (Shigeno *et al.*, 1985). Briefly, all rats underwent subtemporal subperiosteal craniectomy (with intact zygoma) and exposure of the main trunk of MCA under 16 × magnification of an operating stereomicroscope (M351, Leica Instruments, Nussloch, Germany). The exposed artery was electrocoagulated close to its origin at the junction with the olfactory branch. Each rat was allowed to breath spontaneously and body temperature was maintained at 37°C (36.5–37.5°C) with a homeothermic heating blanket. All necessary care was taken to perform surgery under sterile conditions.

The experimental groups were:

1. Control rats which have been anaesthetized but not operated (both brain hemispheres)
2. Sham-operated rats which have been anaesthetized and sham-operated (craniectomy and exposure of the main trunk of left MCA).
3. Ischaemic rats which have been anaesthetized and operated (craniectomy and permanent occlusion of the left MCA).

Drug administration

MK-886 was dissolved in a saline solution with 10% DMSO, and administered in the femoral vein at two doses (0.3 mg kg^{-1} or 2 mg kg^{-1}). MK-801 was dissolved in a saline solution and given at the dose of 3 mg kg^{-1} i.p.; SCH58261 was dissolved in saline solution additioned with 10% DMSO and administered i.p. at the dose of 0.01 mg kg^{-1}. Rats were given drugs or their vehicle in a volume of 5 ml kg^{-1} within 10 min after the pMCAo.

Infarct size analysis

Rats were sacrificed by decapitation 24 h after pMCAo in order to have the ischaemic damage completly developed (Kirino *et al.*, 1988). The brains were rapidly removed and fixed in Carnoy (60% EtOH, 30% chloroform, 10% acetic acid glacial). Infarct volume was determined on paraffin-embedded coronal slices (10 μm) stained with cresyl violet to determine the cortical and striatal damage. Sections were sampled at a distance of 1 mm starting from 3.2 mm from bregma, following a rostro-caudal direction, for eight levels. Total brain and infarct areas were measured by using an image analyser (Image-Pro Plus, Media Cybernetics, MD, U.S.A.). The volume of infarction was calculated with trapezoid's estimator of morphometric volume (Rosen & Harry, 1990) and corrected for oedema. The amount of ischaemic damage was expressed in absolute values (mm^3).

Oedema was evaluated indirectly as percentage increase of the ischaemic hemisphere volume with respect to the control hemisphere, using the following formula: $[(V_{ipsi} - V_{contra})/V_{ipsi}] \times 100$, where V_{ipsi} = volume of the ipsilateral (ischaemic) hemisphere, V_{contra} = volume of the contralateral (healthy) hemisphere.

LT extraction and analysis

Cerebral tissue Male rats were sacrificed by decapitation at different times (1, 4, 6 and 24 h) after the operation. The head was immediately transferred on ice; brain cortex and hippocampus, both ipsi- and contralateral with respect to the operated hemisphere, were quickly removed, weighed and homogenized in ice-cold absolute EtOH (1 : 3, w v^{-1} for the cortex; 1 : 9, w v^{-1} for the hippocampus). In order to have detectable amounts of i-cysteinyl-LTs under all conditions,

brain areas from two animals were combined and the samples were centrifuged at $10,000 \times g$ for 15 min at 4°C. The supernatants were removed and, after addition of ^{3}H-LTC$_4$ (50,000 d.p.m./sample) for recovery calculation, they were stored at −20°C until analysis and in any case not longer than 15 days.

For LT analysis, each sample was diluted with Ultrapure H_2O to obtain a final EtOH concentration of 15% and extracted using a Sep-Pak C18 solid phase cartridge, previously washed with 3 ml MeOH and 3 ml H_2O. The column was eluted with 3 ml hexane (discarded), then with 1 ml EtOAc to elute LTB_4 and finally with 1 ml MeOH to elute cysteinyl-LTs.

MeOH fractions were separately dried and reconstituted in buffer (0.1 M K_2HPO_4, 0.1 M KH_2PO_4, 1.5 mM NaN_3, 0.4 M NaCl, 1 mM EDTA, 1 g l^{-1} bovine serum albumin) just before enzyme immunoassay (Pradelles *et al.*, 1985; 1990). Solid phase EIA was performed on 96-well microplates with the Titertek apparatus (Flow Laboratories, Helsinki, Finland), using an antibody with a high (50–90%) cross-reactivity between LTC_4, LTD_4 and LTE_4, in order to be able to measure all the cysteinyl-LTs (immunoreactive cysteinyl-LTs, i-cysteinyl-LTs). The antibody displayed negligible interaction with the prostanoids and fatty acids (cross-reactivity <0.01 and 0.12%, respectively). The detection limit was 10–15 fmol. The total recovery for cysteinyl-LTs was approximately 50%.

Samples containing the radioactive standards, but not tissue, were processed in parallel with the others and represent the blank of the procedure.

Lung parenchyma Macroscopically normal human lung tissue was obtained at the time of resection. Tissue fragments of approximately 100 mg each were incubated overnight in gassed Tyrode's buffer (mM): NaCl 140, $MgCl_2$ 0.5, KCl 2.7, $CaCl_2$ 1.7, NaH_2PO_4 0.36, glucose 5, $NaHCO_3$ 12, pH 7.4 at 25°C. The next day, the lung fragments were washed and resuspended in Tyrode's buffer 1 : 10 w v^{-1}. After 15 min at 30°C, they were treated with either the drugs under study (30 nM SCH 58261, 10 μM MK-801, 1 μM MK-886) or their vehicle; 15 min later, they were challenged with A23187 for 20 min. The tissue was then eliminated and the supernatant was frozen. The samples were analysed for LT content as described above.

Data analysis

LT levels are expressed as fmol g^{-1} of tissue, mean ± s.e. Infarct volume and percentage of infarction are presented as mean ± s.e. Statistical evaluation was carried out by analysis of variance, one or two way ANOVA, according to the experimental design. A P value <0.05 was considered to be statistically significant.

Results

Basal levels of i-cysteinyl-LTs in cerebral cortex

In control animals, which had undergone anaesthesia but no surgery, there were detectable levels of i-cysteinyl-LT after 2 h (425 ± 85 fmol g^{-1} tissue, $n=4$; Figure 1). These levels were not different from those in contralateral cortices from both ischaemic and sham-operated animals. The two latter conditions yielded i-cysteinyl-LT levels not significantly different from one another at any time up to 24 h after pMCAo; for this reason, such values were pooled at each time point (Figure 1) and compared with the levels in ipsilateral cortices from sham-operated animals. As shown in Figure 1, in the ipsilateral cortices the surgical procedure caused i-cysteinyl-LT levels to be significantly higher ($P<0.05$) than in the contralateral areas at 2 and 4 h after occlusion. Therefore, the values of the ipsilateral cortices of sham-operated animals, which include the basal production, were taken as reference values to evaluate the effect of ischaemia.

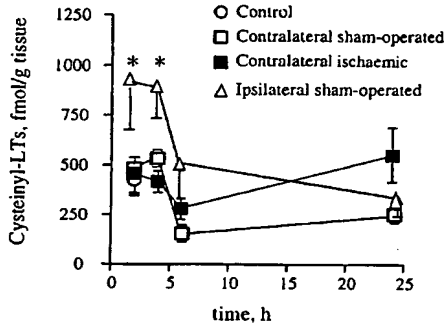

Figure 1 Time course of the variation of cysteinyl-LT levels in the contralateral cortices from sham-operated and ischaemic animals and in the ipsilateral cortices from sham-operated animals. Data are means ± s.e.mean, $n=3-5$. (*$P<0.05$ *vs* contralateral cortices). Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test.

Cysteinyl-LT levels after pMCAo

Cysteinyl-LT levels in cerebral cortex following pMCAo were different from values obtained in sham-operated animals. Following ischaemia, i-cysteinyl-LT levels peaked at 4 h and at this time point were approximately four times higher than reference values ($P<0.01$) (Figure 2).

On the contrary, the levels of i-cysteinyl-LTs in the hippocampus, an area which is not involved in ischaemic damage upon pMCAo (Shigeno *et al.*, 1985), in ischaemic animals (3970 ± 1009 fmol g^{-1} tissue, $n=3$) were not significantly different from those either in contralateral areas (2006 ± 197 fmol g^{-1} tissue, $n=3$) of the same animals or in hippocampi of sham-operated ones (2908 ± 640 fmol g^{-1} tissue, $n=3$).

Pharmacological modulation of i-cysteinyl-LT levels after pMCAo

When rats were treated with the NMDA receptor antagonist MK-801 (3 mg kg^{-1} i.p.) after pMCAo, ischaemia-induced i-cysteinyl-LT increase measured 4 h after the occlusion was reduced by 92.4% ($P<0.01$, Figure 3). The adenosine A_{2A} receptor antagonist SCH 58261 (0.01 mg kg^{-1} i.v.) showed a trend to reduce the increase in i-cysteinyl-LT levels (−48%) after ischaemia, but this inhibition did not attain statistical significance. The 5-LOX inhibitor MK-886 at both doses (0.3

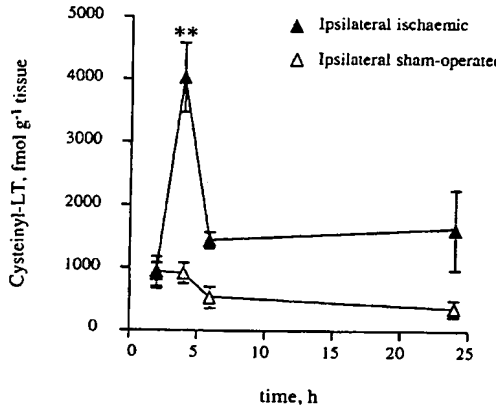

Figure 2 Time course of the variation of cysteinyl-LT levels in the ipsilateral cortices from ischaemic animals compared with reference values (ipsilateral sham-operated cortices). Data are means±s.e.mean, $n=4-9$. (**$P<0.01$ *vs* sham-operated cortices). Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test.

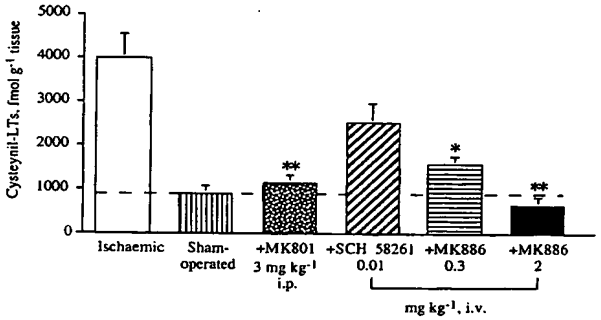

Figure 3 Variation of cysteinyl-LT levels in ischaemic cortices induced by *in vivo* administration, of MK-801 (3 mg kg^{-1} i.p.), SCH 58261 (0.01 mg kg^{-1} i.v.) and MK-886 (0.3 and 2 mg kg^{-1} i.v.). Cysteinyl-LT levels were assayed 4 h after pMCAo. Inhibition was evaluated by taking cysteinyl-LT formation in ipsi-lateral sham-operated cortices as basal value. Data are means±s.e.mean, $n=4-10$. (*$P<0.05$ and **$P<0.01$ *vs* ischaemic cortices). Statistical analysis was performed out by one-way ANOVA followed by Bonferroni's test.

and 2 mg kg^{-1} i.v.) under the same conditions inhibited i-cysteinyl-LT increase by 78% ($P<0.05$) and 100% ($P<0.01$), respectively (Figure 3). Administration of drug vehicles did not modify i-cysteinyl-LT levels in ischaemic cortices.

In order to evaluate whether MK-801 and SCH 58261 were able to directly inhibit i-cysteinyl-LT formation, we measured the effect of MK-801 (10 μM) and SCH 58261 (30 nM) in comparison to MK-886 (1 μM) in human lung parenchyma, a tissue less rich in both NMDA and adenosine A_{2A} receptors. The ratio of SCH 58261 or MK-801 concentrations to MK-886 concentration was higher than those used *in vivo*. In this preparation, the challenge with 10 μM A23187 caused the production of 333±25 fmol mg^{-1} tissue. Neither MK-801 nor SCH 58261 inhibited i-cysteinyl-LT production significantly (673±100 and 472±59 fmol mg^{-1} tissue, respectively), whereas MK-886 induced an almost complete inhibition (37±1 fmol mg^{-1} tissue, −92%, $P<0.01$).

Effects of the leukotriene synthesis inhibitor MK-886 on infarct size after pMCAo

The permanent occlusion of left MCA resulted in a reproducible ischaemic damage within the territory of the artery, i.e. in the dorsolateral cortex and in the neostriatum, as well as an increase in the volume of the lesioned hemispheres, representing the occurence of oedema. The administration of MK-886 at the lowest dose (0.3 mg kg^{-1} i.v.) 10 min after the pMCAo did not significantly reduce the volume of ischaemic brain damage in the cortex and striatum (infarct volume: total: 84.8±9.5 *vs* 77.3±9.1 mm^3; cortical: 60.6±7.6 *vs* 54.9±7.2 mm^3; striatal: 24.2±3.0 *vs* 21.7±2.9 mm^3; ($n=10-11$)). At the higher dose (2 mg kg^{-1} i.v.), MK-886 significantly reduced total infarct volume by 25% and cortical infarct volume by 30% ($P<0.05$) (Figure 4), whereas striatal infarct volume was not significantly affected by drug treatment.

On the contrary, MK-886 did not significantly decrease oedema (oedema: vehicle 16%±2.5; MK-886 10%±2), although a tendency toward inhibition was observed.

No changes in physiological parameters, such as mean arterial blood pressure and heart rate, were observed during surgical and pharmacological treatments (data not shown).

Discussion

The present study shows that i-cysteinyl-LT levels are increased in rat brain cortex in a model of permanent focal ischaemia, upon middle cerebral artery occlusion (pMCAo). Previous evidence of increased LT formation in brain ischaemia had been obtained only in models of global ischaemia (Dempsey *et al.*, 1986a,b; Mabe *et al.*, 1990; Minamisawa *et al.*, 1988; Moskowitz *et al.*, 1984), which, as already mentioned, are not as relevant to human stroke as the focal ischaemia model.

Treatment with the 5-lipoxygenase inhibitor MK-886 (Gillard *et al.*, 1989) significantly inhibited ischaemia-induced

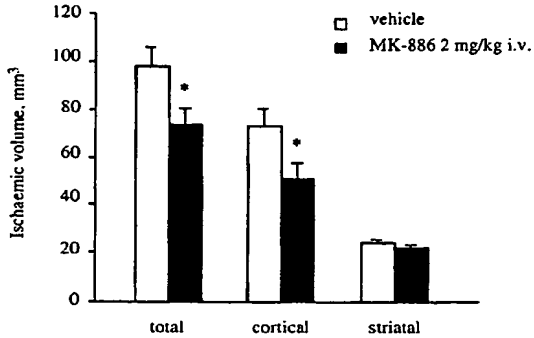

Figure 4 Total, cortical and striatal infarct volume 24 h after pMCAo in rats treated with either MK-886 (2 mg kg^{-1} i.v.) or vehicle after pMCAo. Data are means±s.e.mean, $n=10-13$. (*$P<0.05$ *vs* vehicle; statistical analysis was performed by two-way ANOVA followed by Dunnett's test for multiple comparison).

i-cysteinyl-LT formation at both doses tested. In particular, the higher dose (2 mg kg^{-1} i.v.) completely abolished LT formation. Although no direct data exist on the ability of this drug to cross blood-brain barrier, our results indicate that such passage very likely occurs, at least under conditions where the blood brain barrier is partially altered. The effect of MK-886 was compared with that of the known neuroprotective drugs MK-801, a glutamate NMDA receptor antagonist, and SCH 58261, an adenosine A_{2A} antagonist, at doses shown to be effective in decreasing the infarct size (Hatfield *et al.*, 1992; Monopoli *et al.*, 1998). Unexpectedly, MK-801 significantly decreased i-cysteinyl-LT levels suggesting that stimulation of NMDA receptors leads to activation of 5-LOX in the brain. To our knowledge, this is the first evidence that the glutamate NMDA receptor is associated with the formation of cysteinyl-LTs.

Cerebral ischaemia induced by pMCAo in the rat has gained increasing acceptance as a model of focal infarction in humans (Shigeno *et al.*, 1985; Tamura *et al.*, 1981). In this ischaemia model, surgery and the subsequent tissue manipulation induces an increase in the levels of the inflammatory mediators cysteinyl-LTs, as demonstrated by their levels in sham-operated animals. However, such levels are significantly higher in the ipsilateral cortices of ischaemic rats. This indicates that the ischaemic event *per se* triggers the formation of a considerable amount of i-cysteinyl-LTs. The reperfusion period following an ischaemic event is considered to be the major player for the inflammatory response in the brain infarcted areas. There is evidence that also in pMCAo a strong inflammatory reaction occurs, accompanied by cytokine release and inflammatory cells infiltration (Garcia *et al.*, 1994; Liu *et al.*, 1993; 1994; Schroeter *et al.*, 1994; Stroemer & Rothwell, 1998).

In our model, the i-cysteinyl-LT levels peak at 4 h after pMCAo and rapidly decline thereafter. Such relatively rapid increase in levels suggests that i-cysteinyl-LTs are not formed by infiltrating cells. Indeed, the influx of monocytes and neutrophils becomes relevant at a much later time (Zhang *et al.*, 1994; Zhang & Chopp, 1997). It is likely that i-cysteinyl-LTs are synthesized by resident brain cells, although the precise source has not yet been fully clarified. Anterior pituitary cells and astroglial cells in culture are able to form cysteinyl-LTs (Hartung & Toyka, 1987; Kiesel *et al.*, 1991; Petroni *et al.*, 1991; Seregi *et al.*, 1990) and more recently, it has been shown that purified microglial cells are mainly involved, rather than astrocytes (Matsuo *et al.*, 1995). On the other hand, the release of glutamate following pMCAo peaks much earlier (about 2 h post ischaemia induction) (Matsumoto *et al.*, 1992; Melani *et al.*, 1999) and thus it could be an up-stream event with respect to cysteinyl-LT formation.

Because cysteinyl-LTs are well-known inflammatory mediators, able to cause oedema (Bochnowicz & Underwood, 1995; Dahlen *et al.*, 1981; Evans *et al.*, 1989), the effect of MK-886 on ischaemia-induced oedema was evaluated. However, MK-886 had no effect on ischaemia induced oedema. In a model of global ischaemia, the administration of other 5-LOX inhibitors (nordihydroguaiaretic acid and AA-861) actually reduced oedema to a significant extent (Dempsey *et al.*, 1986a; Mabe *et al.*, 1990; Watanabe & Egawa, 1994). These first-generation lipoxygenase inhibitors possess also anti-oxidant activity (Steinhilber, 1999), which might contribute to reduction of oedema independently from 5-LOX inhibition. This does not apply to the more specific inhibitors, such as MK-886, which inhibit 5-LOX activity by interacting with FLAP (Five Lipoxygenase Activating Protein) (Dixon *et al.*, 1990). The use of a different inhibitor together with the type of analysis we applied, based on an indirect evaluation of water content, could explain the discrepancy with our results.

Interestingly, MK-886, at the dose of 2 mg kg^{-1}, significantly reduced the infarct size in the cerebral cortex (about 30%). Such an effect is comparable with that obtained with the reference compounds MK-801 (−39%) and SCH 58261 (−30%) in our same laboratories (Monopoli *et al.*, 1998). Ischaemic damage in striatum was marginally influenced by the administration of the drug. In the *core* region (caudate putamen and lower frontoparietal somatosensory cortex), where the reduction of blood flow is more severe, energy failure occurs rapidly, followed by neuronal death. In surrounding at-risk areas, mainly frontal and parietal cortex, neurones remain viable and may be salvaged by restoration of blood flow. The neuroprotective effects of MK-886 was most prominent in these cortical areas.

In our experimental conditions, MK-886-induced neuroprotection was observed only at a dose which inhibited i-cysteinyl-LT formation completely. This might reflect the multifactorial nature of the ischaemic damage, where other mediators besides cysteinyl-LTs, possibly released with a different time-course, might play a role. An alternative explanation might reside in the very high potency of these lipid mediators, such that, with a partial inhibition, concentrations high enough to be fully active are mantained, as previously observed with another inhibitor of LT formation, loratadine (Letari *et al.*, 1994). Accordingly, MK-801, a neuroprotective agent, reduced i-cysteinyl-LT levels more than 90%. To the contrary, SCH 58261 which significantly decreases the infarct size at the same dose tested in the present study (Monopoli *et al.*, 1998), might act through mechanisms which only partially involve cysteinyl-LTs. In our experiments it did not lower i-cysteinyl-LT levels significantly.

Although unidentified lipoxygenase products have been suggested to be neurotoxic (Baran *et al.*, 1994; Chen *et al.*, 1995), this is the first *in vivo* evidence that cysteinyl-LTs might be involved in the development of ischaemia-induced neurotoxicity, as evaluated by infarct size analysis. Thus, this suggests that anti-LT drugs might have neuroprotective properties.

With regard to neuroprotection, there are some considerations of interest: first, so far all the studies on the inhibition of cerebral leukotriene synthesis had been performed using the global model of cerebral ischaemia and with oedema formation as the only end-point (Mabe *et al.*, 1990). In addition, a neuroprotective action of 5-LOX inhibitors had been previously shown only in *in vitro* models of traumatic or hypoxic neuronal injury (Girard *et al.*, 1996; Wallis & Panizzon, 1993). To our knowledge, this is the first demonstration that the inhibition of leukotriene synthesis is able to decrease the infarct size, although we cannot exclude that, in our model, neuroprotection derives from the reduction of brain oedema (which however did not attain statistical significance), due to lower levels of cysteinil-LT.

Finally, the 5-LOX inhibitor MK-886 provided neuroprotection when administered post-ischaemia and by an acute

injection, aspects relevant to possible therapeutic use (Jonas *et al.*, 1997).

In conclusion, our results suggest that an increase in cysteinyl-LT levels following cerebral ischaemia is mainly associated with the activation of the NMDA receptor by glutamate, although we cannot exclude that other mechanisms, activated by the ischaemic event, such as spreading depression, may be involved in LT production. From these findings, it appears that these lipid mediators may play an important role in development of brain damage. In view of this, potential neuroprotective properties of anti-LT compounds need to be further investigated.

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